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(54) Title: INSECT ECDYSTEROID RECEPTORS

(57) Abstract

An ecdysteroid receptor from *Lucilia cuprina* has been isolated, sequenced and expressed. Assays for detecting ligands to the receptor are disclosed. The ligands and receptors are useful in modulating insect development.

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INSECT ECDYSTEROID RECEPTORS

FIELD OF THE INVENTION

This invention relates to insect ecdysteroid receptors. In particular, it relates to ecdysteroid receptors from the Australian blowfly *Lucilia cuprina.*

BACKGROUND OF THE INVENTION

Flies, including blowflies, are carriers of disease affecting humans and other animals. These animals include economically important stock such as sheep, cattle, poultry and horses.

Of the many known species of blowfly a few are responsible for serious conditions such as flystrike. This condition is caused by the larvae of blowflies feeding on the skin of an animal. Sheep are particularly susceptible to flystrike. Flystrike losses are currently estimated to cost about $40 million annually in New Zealand alone. These losses arise through lost production in meat and wool, pelt damage, deaths and associated production and treatment costs. *Lucilia cuprina,* or the Australian blowfly, is the main cause of flystrike in New Zealand.

Current fly population control methods include removal of flies from the environment by trapping or reducing breeding sites (eg. carcases); and managing stock through husbanding methods such as shearing, crutching, dagging, wound treatment and in some cases mulesing. These control methods are generally only partially effective.

An alternative or additional method is chemical treatment of sheep including methods such as dipping, showering, back-line treatments and the like. Continued use of chemicals is subject to a number of disadvantages. Firstly, blowflies develop tolerance to chemicals over time producing insecticide resistant populations. Secondly, chemical residues pose environmental hazards from discharged dips and scour products, as well as health concerns. In particular, wool can have high insecticide residues, exposing shearers and other wool handlers to risk.

Biological control presents an alternative means of insect control which is potentially more effective and specific than current methods, as well as reducing dependence on chemical insecticides.

One approach to biological control requires the identification and characterisation of insect genes or gene products which may serve as suitable targets for insect control agents. Insect steroid hormones and receptors are likely candidate targets for control agents. Of particular interest are the ecdysteroids.

The ecdysteroids are steroid hormones which form part of the endocrine systems of insects. Ecdysones such as 20-OH ecdysone (also known as β-ecdysone) have been shown to control the timing of development in many insects including species of fly.

Pulses, or rises and falls in ecdysone concentration are observed at various stages of fly development. Concentrations of the ecdysone are usually constant during a feeding and growing phase but decline during transition phases such as from egg to larva, larva to pupa and pupa to adult (Ecdysone: from Chemistry to Mode of Action; *Thieme Medical Pub, N.Y.* (1989)).

One studied effect of ecdysone on development is that resulting from a pulse at the end of the last larval stage. This pulse triggers the metamorphosis of the larva to the adult fly.

A genetic regulatory model was proposed to explain the effect of the ecdysone pulse which triggers the larval-to-adult metamorphosis. See Ashburner et al., on "The Temporal Control of Puffing Activity in Polytene Chromosomes," *Cold Spring Harbor Symp. Quant. Biol.* 38:655-662 (1974). This model proposed that ecdysone interacts reversibly with a receptor protein, the ecdysone receptor, to form an ecdysone-receptor complex. These complexes recognise specific DNA sequences called ecdysteroid response elements (EcRE) scattered throughout the genome. These complexes would directly induce the transcription of a small set of "early" genes postulated to encode regulatory proteins that induce the transcription of a second set of "late" genes. The model thus defines a genetic regulatory hierarchy of three ranks, where the ecdysone receptor gene is in the first rank, the early genes in the second rank and the late genes in the third. The receptor-DNA interaction is complex and receptor affinity for EcRE depends on its sequence and its tertiary folding as well as its surrounding chromatin structure.

Similar genetic regulatory hierarchies may also determine the metamorphic changes in development of the imaginal tissues that are also targets of ecdysone, as well as the changes in tissue development induced by the pulses of ecdysone that occur at other developmental stages.

Various structural data have also been derived from vertebrate steroid and other lipophilic receptor proteins. A "superfamily" of such receptors has been defined on the basis of their structural similarities. See Evans, "The Steroid and Thyroid Hormone Receptor Superfamily," *Science* 240: 889-895 (1988); Green and Chambon, "Nuclear Receptors Enhance Our Understanding of Transcription Regulation" *Trends in Genetics* 4:309-314 (1988).

The structure of the superfamily of receptors reveals the existence of five receptor domains, for convenience identified herein as regions A/B, C, D, E and F. These five domains include a DNA binding domain (C) and a hormone binding domain (E). The DNA binding domain is configured into two "zinc-binding fingers", with a zinc ion in each finger forming a tetrahedral coordination complex with cysteine residues.

Where their functions have been defined, these superfamily receptors, complexed with their respective hormones, regulate the transcription of their primary target genes, as proposed for the ecdysone receptor in the above model.

Ecdysteroid receptors from Drosophila melanogaster were described by Handler et al. *Mol Cell Endo* 63:103-9, 1989, Koelle et al.; *Cell*, 67:59-77, 1991 and in US Patent Number 5,514,578. Corresponding receptors from *Aedes aegypti* (AaEcR) and Chironomus tentans (CtEcR) have been described by Cho et al.; and Imhof et al.; in *Insect Biochem Molec. Biol.* 25:19-27, 1995 and 23:115-124, 1993 respectively. Ecdysteroid receptors for the blowfly Calliphora vicina were characterized by Lehmann et al., *Eur J. Biochem* 181:577-82, 1989.

There is still an absence of detailed information on the structure and sequence of steroid receptors from other insects, particularly disease carrying insects and including the Australian blowfly. Detailed sequence information on the genes encoding these receptors would aid the identification of new molecules which may act as agonists or antagonists of the receptor; would allow the receptor itself to be used as a component of an inducible expression system for regulating the expression of a gene of interest; would facilitate the understanding of the tertiary structure and spatial interactions between a ligand and the receptor that will potentially direct the new designs for effective insecticides; would provide a basis for the study of the steroid action and the cascade events in the signal transduction pathway; would facilitate the production of anti-sense RNA or antibodies which could be used in a similar system for regulating the expression of genes; the ultimate goal being to disrupt or regulate the processes involved in insect development. For example by means of transposon-mediated transformation.

The applicant has now identified an ecdysteroid receptor for the Australian blowfly *Lucilia cuprina*. It is broadly to this receptor that the present invention is directed.

SUMMARY OF THE INVENTION

Accordingly, in one aspect, the present invention may broadly be said to consist in an isolated *Lucilia cuprina* ecdysteroid receptor (LcEcR) polypeptide which has the amino acid sequence set out in Figure 4, or a variant thereof having substantially equivalent receptor activity thereto.

In a further aspect, the present invention provides an isolated peptide which comprises or consists of the amino acid sequence for one or more of receptor domains A/B, C, D, E or F as set out in Figure 4.

Preferably, the peptide comprises the amino acid sequences for domain C or E.

Conveniently, the receptor polypeptide of the invention is obtained by expression of a DNA sequence coding therefore in a host cell or organism.

In a further aspect, the present invention provides an isolated nucleic acid molecule including a receptor polypeptide or peptide of the invention.

This nucleic acid molecule can be an RNA or cDNA molecule but is preferably a DNA molecule.

Also provided by the present invention are recombinant expression vectors which contain a DNA molecule of the invention, and hosts transformed with the vector of the invention capable of expressing a polypeptide or peptide of the invention.

In a further aspect, the present invention provides an inducible expression system comprising:

(a) a controlling element responsive to a ligand of an ecdysteroid receptor polypeptide or peptide of the invention;

(b) a desired gene for expression, operably linked to the controlling element; and

(c) an ecdysteroid receptor which can bind to the controlling element,

In a still further aspect, the invention provides a method of producing a polypeptide or peptide of the invention comprising the steps of:

(a) culturing a host cell which has been transformed or transfected with a vector as defined above to express the encoded polypeptide or peptide; and

(b) recovering the expressed polypeptide or peptide.

An additional aspect of the present invention provides a ligand that binds to a polypeptide or peptide of the invention. Most usually, the ligand is a phytoecdysteroid or an antibody or antibody binding fragment. Such ligands also form a part of this invention.

In further aspects, the present invention provides methods of assaying samples for the presence of ligands; test kits suitable for use in such assays; test kits comprising the inducible expression system of the invention, methods of modulating the development of insects by administering selected products of the invention to an insect, and compositions and agents useful in such methods.

In one aspect there is provided a method for screening for ecdysteroid ligands, the method comprising:

(a) contacting an ecdysteroid responsive element (EcRE), an ecdysteroid receptor (EcR) and a reporter system with a sample;

(b) detecting the presence of any bound, reported ecdysteroid receptor.

While the invention is broadly as defined above, it will be appreciated by those persons skilled in the art that it is not limited thereto and that it also includes embodiments of which the following description gives examples.

In particular preferred aspects of the invention will be described in relation to the accompanying drawings in which:

Figure 1 is schematic diagram depicting the cloning strategy, structure and function of the ecdysteroid receptor from *Lucilia cuprina*.

Figure 2 is a pileup analysis of LcEcR, AaEcR, CtEcR and DmEcR. The DNA binding domain in bold between 317-382. The hormone binding domain is in bold between 492-713. Degenerated primers are underlined with arrow indicating forward primers and with arrow indicating reverse primers.

Figure 3 depicts the results of the 557 BP Fragment amplification of LcEcR by RT-PCR. The PCR products were electrophoresed in a 1% agarose gel and 1 x TAE buffer. DNA bands were visualised by staining with EtBr. Lane 1 shows Lambda phage Hind III digestion markers. Lanes 2-4 are the first-run PCR products with 503 and 302 primers as a set. Lanes 5-7 are the second-run PCR with 503 and 304 primers as a set. Lanes 2 and 5 are positive controls using DM RNA. Lanes 4 and 7 are negative controls without adding any RNA. Lanes 3 and 6 are PCR products from LcRNA. Lane 6 shows a DNA band with a size of 557bp.

Figure 4 represents both the deduced amino acid sequence of the open reading frame of the LcEcR polypeptide of the invention and the nucleotide sequence coding therefore. The domains are identified as follows:

Domain	Nucleotides	Amino Acids
A/B	1 - 900	1 - 300
C	901 - 1098	301 - 366
D	1099 - 1359	367 - 453
E	1360 - 2022	545 - 674
F	2023 - 2271	675 - 757

The DNA and hormone binding domains (domains C and E respectively) are in bold. Primers 511 and 512 and 3'end were boxed and labelled. The nuclear localisation signals (NLS) are underlined. The helix-turn-zipper motif is underlined with alternating single and double lines.

Figure 5 depicts the results of the 1.8kb fragment amplification of LcEcR by 3'RACE. The PCR products were shown in 1% agarose gel. Lane 1 shows Lambda Hind III digestion markers. Lane 2 is on first-run PCR product with primer 511 and PCR anchor as a set. It shows a 1.8 kb band.

Figure 6 depicts the results of the 2.8 kb fragment of the open reading frame of LcEcR by RT-PCR. The PCR products were shown in 1% agarose gel. Lane 11 shows Lambda Hind III digestion markers. Lane 2 is first- run PCR product with primers ORF1 and PCR anchor as a set. Lane 3 is the second run PCR product with primers ORF1 and 3'end as a set. It shows a 2.8 kb band.

Figure 7 depicts the baculoviral vector system, *Trichophsia ni* nuclear polyhedrosis virus, TnNPV, and the vector incorporating LcEcR cDNA, TnNPV-EcR.

Figure 8
Western blot analysis of the recombinant baculovirus TnNPV-EcR. After 72 hours post infection, Tn-5B1 cells infected with wild-type TnNPV and TnNPV-EcR were lysed and processed and electrophoresed on a 7.5% SDS-PAGE gel. The gel was then transferred onto nitrocellulose blotting membrane. The first antibody (anti HSV-gD with 1:5000 dilution) was used and then the second antibody (anti-mouse IgG) was applied to detect HSV-gD epitope. Right Lane shows Tn-5B1 cells infected with wild-type TnNPV. Left lane shows Tn-5B1 cells infected with TnNPV-EcR.

Figure 9
Restoration of the ecdysone response in an EcR-deficient Drosophila cell line L57-3-11. EcR deficient Drosophila L57-3-11 cells were transfected with control plasmid pUC19, with the ecdysone response reporter plasmid pEcR/Adh/βgal alone or with the reporter plasmid plus an LcEcR expression plasmid, pAct/EcR. Transfected cells were left untreated, or treated with 20-hydroxyecdysone and cell extracts were assayed for β-gal activity.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides an isolated *Lucilia cuprina* ecdysteroid receptor (LcEcR) polypeptide which has the amino acid sequence set out in Figure 4, or a variant thereof having substantially equivalent receptor activity thereto.

The open reading frame of the LcEcR contains 757 amino acids with a molecular weight of approximately 84 kD. The deduced amino acid sequence of LcEcR exhibits a high level (up to 72%) identity to DmEcR, AaEcR, and CtEcR.

The term "variant" as used herein refers to a polypeptide wherein the amino acid sequence exhibits substantially 75% or greater homology with the amino acid sequence set out Figure 4. The variant may be arrived at by modification of the native amino acid sequence by such modifications as insertion, substitution or deletion of one or more amino acids.

In a related aspect, the invention also provides an isolated peptide which comprises or consists of the amino acid sequence for one or more of receptor domains A/B, C, D, E or F shown diagrammatically in Figure 1 and with the sequences set out in Figure 4, or fragments thereof.

Domain C is a 66 amino acid sequence coding for the DNA binding domain. As discussed above, the DNA binding domain comprises two C_2C_2 zinc fingers which are responsible for the recognition and binding of the receptor to an ecdysteroid response element. These are specific DNA sequences that are recognised by the receptors. Two amino acid motifs within the DNA binding domain confer receptor specificity for a particular ecdysteroid response element. These amino acid motifs are depicted as the proximal box (P-box) and the distal box (D-box) in Figure 4.

In a preferred embodiment of the present invention the peptide of the invention comprises or consists of the amino acid sequence for the P-box and/or the D-box of domain C. Domain E shown in Figure 1 comprises the 221 amino acid hormone-binding domain which lies to the carboxy-terminal side of the DNA-binding domain. The hormone binding domain is responsible for the affinity binding of the ecdysteroid hormone to the receptor.

As the reader will appreciate from Table 1 below, the amino acid sequence for domains C and E exhibit a high level of identity between DmEcR, AaEcR and CtEcR.

Table 1: Percentage of the identity between LcEcR (U75355), DmEcR, AaEcR and CtEcR

	Whole ORF	A/B domain	DNA binding domain(C)	D domain	Hormone binding domain (E)	F domain
U75355	95	100*	100	100	99.5	55
DmEcR	70	52	95	83	96	27
AaEcR	72	57	97	67	89.6	32
CtEcR	64	16	97	60	76	31

*: LcEcR A/B domain has 2 amino acid deletions at 177, 178 positions and 3 amino acid insertions at 135,136 and 152 positions

In addition, the ecdysteroid receptor possesses a unique amino-terminal domain through which contact is made with other transcription factors (region A/B in Figure 1), although other domains also interact with regulatory proteins.

Furthermore, the ecdysteroid receptor, being the nuclear transcriptional factor, possesses a nuclear localisation signal (NLS, region D in Figure 1), which are short amino acid sequences that modulate nuclear transfer. Two potential NLS's, MKRREKK and QKEKDKI, were found in this hinge domain.

Preferably, the peptide comprises the amino acid sequence for domain C or E.

The reader will appreciate that modifications of the polypeptides and peptides of the invention are possible. The production of peptide fragments is also well within the capabilities of an art skilled worker.

The polypeptide and peptides of the invention can be prepared in a variety of ways. For example, they can be produced by isolation from natural source, by synthesis using any suitable known techniques (such as by stepwise, solid phase, synthesis described by Merryfield (1963), *J.Amer.Chem.soc.* Vol 85:2149-2156 or as preferred, through employing recombinant DNA techniques.

The variants of both the polypeptide and peptides can similarly be made by any of those techniques known in the art. For example, variants can be prepared by site-specific mutagenesis of the DNA encoding the native amino acid sequence as described by Adelman et al. *DNA* 2:183 (1983).

Where it is preferred, recombinant techniques used to produce the polypeptide or peptide of the invention, the first step is to obtain DNA encoding the desired product. Such DNA comprises a still further aspect of this invention.

The DNA of the invention may encode a native or modified polypeptide or peptide of the invention or an active fragment thereof. In its preferred forms, the DNA comprises at least nucleotides 901-2022 of the sequence of Figure 4, or the nucleotide sequence extending from nucleotides 901-1098 (domain C) or 1360-2022 (domain E) of the sequence of Figure 4.

The DNA can be isolated from any appropriate natural source or can be produced as intron free cDNA using conventional techniques. DNA can also be produced in the form of synthetic oligonucleotides where the size of the active fragment to be produced permits. By way of example, the Triester method of Matteucci et al. *J. Am.Chem.Soc.* Vol 103:3185-3191 (1981) may be employed.

Where desirable, the DNA of the invention can also code for a fusion protein comprising the polypeptide or peptide of the invention and a carrier protein. This carrier protein will generally be cleavable from the polypeptide, peptide or fragment under controlled conditions. Examples of commonly employed carrier proteins are β-galactosidase and glutathione-S-transferase.

As indicated above, also possible are variants of the polypeptide or peptide which differ

from the native amino acid sequence by insertion, substitution or deletion of one or more amino acids. Where such a variant is desired, the nucleotide sequence of the native DNA is altered appropriately. This alteration can be made through elective synthesis of the DNA or by modification of the native DNA by, for example, site-specific or cassette mutagenesis. Preferably, where portions of cDNA or genomic DNA require sequence modifications, site-specific primer directed mutagenesis is employed using techniques standard in the art.

In a further aspect, the present invention consists in replicable transfer vectors suitable for use in preparing a polypeptide or peptide of the invention. These vectors may be constructed according to techniques well known in the art, or may be selected from cloning vectors available in the art.

The cloning vector may be selected according to the host or host cell to be used. Useful vectors will generally have the following characteristics:

(a) the ability to self-replicate;
(b) the possession of a single target for any particular restriction endonuclease; and
(c) desirably, carry genes for a readily selectable marker such as antibiotic resistance.

Two major types of vector possessing these characteristics are plasmids and bacterial viruses (bacteriophages or phages). Presently preferred vectors include plasmids pMOS-Blue, pGem-T and pUC8.

The DNA molecules of the invention may be expressed by placing them in operable linkage with suitable control sequences in a replicable expression vector. Control sequences may include origins of replication, a promoter, enhancer and transcriptional terminator sequences amongst others. The selection of the control sequence to be included in the expression vector is dependent on the type of host or host cell intended to be used for expressing the DNA.

Generally, procaryotic, yeast, insect or mammalian cells are useful hosts. Also included within the term hosts are plasmid vectors. Suitable procaryotic hosts include E. coli, Bacillus species and various species of Pseudomonas. Commonly used promoters such as β-lactamase (penicillinase) and lactose (lac) promoter systems are all well known in the art. Any available promoter system compatible with the host of choice can be used. Vectors used in yeast are also available and well known. A suitable example is the 2 micron origin of replication plasmid.

Similarly, vectors for use in mammalian cells are also well known. Such vectors include well known derivatives of SV-40, adenovirus, retrovirus-derived DNA sequences, Herpes simplex viruses, and vectors derived from a combination of plasmid and phage DNA.

Further eucaryotic expression vectors are known in the art (e.g. P.J. Southern and P.Berg, *J. Mol. Appl. Genet. 1* 327-341 (1982); S. Subramani et al., *Mol.Cell.Biol. 1*, 854-864 (1981); R J. Kaufmann and P.A. Sharp, "Amplification and Expression of Sequences Cotransfected with a Modular Dihydrofolate Reducase Complementary DNA Gene, *J. Mol. Biol. 159*, 601-621 (1982); R J. Kaufmann and P.A. Sharp, *Mol.Cell.Biol. 159*, 601-664(1982); S.I. Scahill et al., "Expressions And Characterization Of The Product Of A Human Immune Interferon DNA Gene In Chinese Hamster Ovary Cells," *Proc. Natl. Acad. Sci. USA. 80*, 4654-4659 (1983); G. Urlaub and L.A. Chasin, *Proc. Natl. Acad. Sci. USA. 77*, 4216-4220, (1980).

The expression vectors useful in the present invention contain at least one expression control sequence that is operatively linked to the DNA sequence or fragment to be expressed. The control sequence is inserted in the vector in order to control and to regulate the expression of the cloned DNA sequence. Examples of useful expression control sequences are the lac system, the trp system, the tac system, the trc system, major operator and promoter regions of phage lambda, the glycolytic promoters of yeast acid phosphatase, e.g. Pho5, the promoters of the yeast alpha-mating factors, and promoters derived from polyoma, adenovirus, retrovirus, and simian virus, e.g. the early and late promoters of SV40, and other sequences known to control the expression of genes of prokaryotic and eucaryotic cells and their viruses or combinations thereof.

Preferred promoters for use herein include Psyn, Pxiv and Ppolh. The dual promoter Psyn and Pxiv is especially preferred as it is a much stronger promoter than any of Psyn, Pxiv or Ppolh alone.

In the construction of a vector it is also an advantage to be able to distinguish the vector incorporating the foreign DNA from unmodified vectors by a convenient and rapid assay. Reporter systems useful in such assays include reporter genes, and other detectable labels which produce measurable colour changes, antibiotic resistance and the like. In one preferred vector, the β-galactosidase reporter gene is used, which gene is detectable by clones exhibiting a blue phenotype on X-gal plates. This facilitates selection. In one embodiment, the β-galactosidase gene may be replaced by a polyhedrin-encoding gene; which gene is detectable by clones exhibiting a white phenotype when stained with X-gal. This blue-white color selection can serve as a useful marker for detecting recombinant

vectors.

Once selected, the vectors may be isolated from the culture using routine procedures such as freeze-thaw extraction followed by purification.

For expression, vectors containing the DNA of the invention to be expressed and control signals are inserted or transformed into a host or host cell. Some useful expression host cells include well-known prokaryotic and eucaryotic cells. Some suitable prokaryotic hosts include, for example, E.coli, such as E. coli, S G-936, E. coli HB 101, E. coli W3110, E.coli X1776, E. coli, X2282, E. coli, DHT, and E. coli, MR01, Pseudomonas, Bacillus, such as Bacillus subtilis, and Streptomyces. Suitable eucaryotic cells include yeast and other fungi, insect, animal cells, such as COS cells and CHO cells, human cells and plant cells in tissue culture.

Expression systems employing insect cells utilising the control systems provided by baculovirus vectors have been described (Miller, D W et al., in *Genetic Engineering* (1986) Setlo W, J K et al., Eds, Plenum Publishing, Vol 8, pages 277-297). These are the presently preferred expression systems.

The baculovirus gene expression vector system is ideal for the expression of LcEcR as it assures appropriate folding and post-translational modifications of LcEcR. The baculoviral vector system, *Trichoplusia ni* nuclear polyhedrosis virus, TnNPV, developed in Dr. Yi Pang's lab in State Key lab for Biocontrol in Zhongshan University in China has three unique advantages: 1) The recombinant virus (TnVPV-EcR, as shown in Figure 7 is designed to express both the viral polyhedrin-encoding gene and a heterologous gene such as LcEcR. These recombinants form polyhedral occlusion bodies which serve as visible markers of recombination. The recombinants can also be used to facilitate oral infection of insect larvae for mass-scale protein productions. 2) The dual promoter, Psyn and Pxiv, which drives LcEcR gene expression, is a much stronger promoter compared with Psyn, Pxiv or Ppolh alone. 3) The β-galactosidase gene expressed by the parental polyhedral inclusion body minus vector, TnNPV-SVIG as shown in Fig.7, can be replaced by polyhedrin-encoding gene by the recombinant virus. Thus the recombinant virus TnNPV-EcR shows white phenotype when stained with X-gal. This blue-white color selection can serve as a useful marker for detecting recombinant viruses.

Depending on the host used, transformation is performed according to standard techniques appropriate to such cells. For prokaryotes or other cells that contain substantial cell walls, the calcium treatment process (Cohen, S N *Proceedings, National Academy of Science,*

USA 69 2110 (1972)) may be employed. For mammalian cells without such cell walls the calcium phosphate precipitation method of Graeme and Van Der Eb, *Virology* 52:546 (1978) is preferred. Transformations into plants may be carried out using Agrobacterium tumefaciens (Shaw et al., Gene 23:315 (1983) or into yeast according to the method of Van Solingen et al. *J.Bact. 130*: 946 (1977) and Hsiao et al. *Proceedings, National Academy of Science*, 76: 3829 (1979).

Upon transformation of the selected host with an appropriate vector the polypeptide or peptide encoded can be produced, often in the form of fusion protein, by culturing the host cells. The polypeptide or peptide of the invention may be detected by rapid assays as indicated above. The polypeptide or peptide is then recovered and purified as necessary. Recovery and purification can be achieved using any of those procedures known in the art, for example by absorption onto and elution from an anion exchange resin. This method of producing a polypeptide or peptide of the invention constitutes a further aspect of the present invention.

Host cells transformed with the vectors of the invention also form a further aspect of the present invention.

Steroid-responsive expression of other genes is also possible using an inducible expression system. In such cases, the steroid receptor gene will typically be co-transfected with a recombinant construct comprising a desired gene for expression operably linked to the steroid-responsive enhancer or promoter element. In this use, a single inducible expression system will typically comprise a combination of (1) a controlling element responsive to a ligand of an ecdysteroid receptor of the invention; (2) a desired gene for expression, operably linked to the controlling element; and (3) an ecdysteroid receptor which can bind to the controlling element. Usually, this system will be within a cell, but an *in vitro* system is also possible. The ecdysteroid receptor will typically be provided by expression of a nucleic acid encoding it, though it need not be expressed at particularly high levels.

Thus, in one preferred embodiment, the system will be achieved through co-transfection of a cell with both the regulatable construct and another segment encoding the ecdysteroid receptor. Usually, the controlling element will be an enhancer element, but it may work in reverse and be used to repress expression. In this embodiment, the ligand for the ecdysteroid receptor will be provided or withheld as appropriate for the desired expression properties.

Since the invertebrate steroid and its receptor are so different from the vertebrate steroid and its receptor and has the low vertebrate toxicity (Spindler-Barth, M., in *Parasitology Research*, 78:89-95, 1992), such an insect steroid-inducible expression system could be useful in human gene therapy where the expression of a particular gene of interest could be controlled in a temporal fashion by administering an insect steroid to an affected individual whose cells have previously been transformed to include an expression system as detailed above. The cells of affected individual can be easily transformed with viral vectors or plasmid vectors carrying this inducible expression system.

In addition, this steroid-inducible expression system could be useful for the production of large amounts of protein. As is commonly known, the production of large quantities of foreign proteins makes some cells unhealthy, which may affect unfavourably the yield of the desired protein. With the help of an inducible expression system, these cells are orchestrated so that they do not express the foreign protein, and therefore are not unhealthy, until an inducing agent (e.g. steroid) is added to the growth medium. In this way, large quantities of healthy cells can be produced and thus induced to produce large amounts of the foreign protein.

A particularly useful genetic construct comprises an alcohol dehydrogenase promoter operably linked to an easily assayable reporter gene, e.g. β-galactosidase. In a preferred embodiment of this construct, a multiplicity of copies of the ecdysteroid receptor is used. For example, operable linkage of controlling elements responsive to ecdysteroid receptor, e.g., EcR to the alcohol dehydrogenase (ADH) promoter, or others as described above, and protein coding sequences for a particular reporter protein, as described above leads to steroid-responsive expression of that protein. This controlling element responsive to the construct provides a very sensitive system for the detection of responsive expression. This will be used in sensitive assays for the presence of a receptor-ligand interaction, allowing for detection of either ligand or receptor or both.

Test kits comprising the inducible expression system of the invention also form a part of the present invention. The test kits may additionally comprise agents including for example, inducing agents, reagents suitable for use with such an expression system, buffers, diluents, standards and other agents known in the art which are commonly employed in such test kits.

In addition, a further aspect of the present invention provides a ligand that binds to a polypeptide or peptide of the invention.

Ligands may be of two functional types. The first functional type of ligand is a molecule which binds to the ecdysteroid receptor and stimulates it in performing its normal function (an agonist ligand). The second functional type of ligand is a molecule which binds to the ecdysteroid receptor and inhibits or prevents it performing its normal function (an antagonist ligand).

These agonists and antagonists can be used to produce insecticidal compositions which interfere with or disrupt insect development.

In one embodiment the ligand may be an antibody or antibody binding fragment raised against the polypeptide or peptide. Such antibodies may be polyclonal, but are preferably monoclonal.

Polyclonal antibodies may be produced according to the method used by Koelle el al.; *Cell* 67:59-77, 1991 incorporated herein by reference. Useful antibody production protocols are outlined in US Patent 5,514,578 incorporated herein by reference. Monoclonal antibodies may be produced by methods known in the art. These methods include the immunological method described by Kohler and Milstein in *Nature* 256:495-497 (1975) as well as by the recombinant DNA method described by Huse et al. *Science* 246:1275-1281 (1989). Any of the assay methods detailed in US Patent 5,514,578 are also incorporated for use herein by reference.

An understanding of the tertiary structure and spatial interactions between the ecdysteroid receptor (especially ligand-binding domains) and its ligand will provide ways to select highly specific ligands which may be bound only by a modification of a natural receptor ligand-binding domain. Also, this knowledge will provide directions for new designs using the combination of ecdysteroid receptors with ligands and methods to design and select peptide mimetics of ligands with high specificity by techniques such as phage differential display.

In another embodiment the ligand may comprise molecules that bind to the polypeptide or peptide of the invention which are derived from natural sources, including plants, animals and insects. Plant extracts which produce phytoecdysteroids are of particular interest.

Accordingly, in a further aspect, the present invention provides a method of assaying samples for the presence of ligands. Assaying processes using polypeptides or peptides as a ligand binding agent or probe are well within the capacity of the art skilled worker.

The selection of the segment to be used as a probe will allow particular functionally associated segments to be isolated. For example, if a segment of the DNA binding domain is used as a probe, other nucleic acid segments encoding other DNA binding domains will be isolated.

It will also be appreciated that the selection of probes highly specific for *Lucilia cuprina*, such as the DNA or hormone binding domains, will provide an opportunity to assay samples in a rapid and highly specific manner by detecting the presence of phytoecdysteroids specific for *Lucilia cuprina*. Currently used bioassays for detection of insecticidal or growth regulatory effects in plant extracts do not distinguish between the effects of phytoecdysteroids and other hormones or toxins. This invention will allow a one-step recognition of specific ecdysteroid activity.

Samples of material to be screened may be prepared in the form of substrate solutions, then exposed to the ligand binding agent or probe. The presence of a ligand binding agent/ligand complex may be detected according to methods also known in the art. Examples of such methods include agglutination, radio immunoassay, fluorescence or enzyme immunoassay techniques. A suitable screening test is an ELISA assay. In this method of the invention it is presently preferred that the hormone binding domain be used as the ligand binding agent.

Besides the above *in vitro* screening assays, the steroid responsive expression system can be employed *in vivo* to screen ligands which have specific ecdysteroid activity by operable linkage of a reporter system to an ecdysteroid responsive element (EcRE) which binds to the ecdysteroid receptor, and where binding is functionally linked to protein induction a sensitive assay for the presence of a ligand or receptor results. For example, a plasmid comprising an EcRE linked with a promoter such as the alcohol dehydrogenase promoter, (ADH) and a reporter gene (for example β-galactosidase (β-gal)) can be transfected with another plasmid encoding the ecdysteroid receptor into the insect cells. In other embodiments, the EcRE, promoter, reporter gene and ecdysteroid receptor may be present in a single plasmid, or a multiplicity of plasmids. In the presence of ligand which could specifically bind to the ecdysteroid receptor, the expression of β-gal activity would be induced. This functional assay can provide a sensitive way to screen ligands that bind to the ecdysteroid receptor.

An example of such a functional assay is provided in Example 6 below.

In an alternative method, cells which do not support ecdysone-responsive transactivation

can be used. For example, some cell lines of mammalian origin such as CV1 do not support ecdysone-responsive transactivation. Therefore, the LcEcR expression vector (pAcT/EcR) and DmUSP expression vector such as CMX-usp (Oro et al., 1990, Yao et al., 1992 both incorporated herein by reference) can be cotransfected into CV1 cells. The reporter plasmid pEcRE/Adh/βgal can also be cotransfected into CV1 cells. In the presence of 20-hydroxyecdysone, LcEcR/DmUSP forms a specific complex with the EcRE in CV1 cells, transactivating the β-galactosidase reporter gene expression. β-gal activity can be readily detected with a fluorescent reagent.

In a further aspect of the invention there is provision for selecting new hormone analogues. In this instance, the hormone binding domain will be used as the ligand binding agent. Such ligand binding methods are disclosed in US 5,514,578 which is specifically incorporated herein by reference.

It will be appreciated by the reader that a further aspect of the invention therefore contemplates the use of the polypeptides or peptides of the invention in the preparation of probes for the detection of other steroid receptors, hormones, or ligands.

In a further aspect the present invention provides test kits suitable for use in such assays. An example of such a test kit is an ELISA assay test kit including a ligand binding agent of the invention.

A still further aspect of the present invention provides methods of modulating the development of insects.

In a first method it is desirable to bring about the over expression of the ecdysteroid protein in insect larvae, particularly blowfly larvae, by the induction of ecdysteroid hormone or its analogues to prematurely turn on the normal developmental moulting process leading to the death of the insect.

Another way in which the moulting hormone activity may be disrupted is by introducing the "anti-sense" RNA acid of the ecdysteroid receptor into an insect. This anti-sense RNA binds to the sense receptor RNA produced by the insect larvae, thereby blocking the receptor protein translation and further signal transduction pathways. This would delay and eventually terminate normal moulting stages in larvae, again leading to death.

Delivery of the ecdysteroid receptor gene or anti-sense RNA to the insect may be achieved by way of vectors described above, either to the larvae or adult insect.

Dissemination through adult flies may also be achieved by placing the vector in a bait. A vector in such a case may be viral. The adult will then have to be capable of transovarial transmission (Smith 1967) so that larvae would be infected through the eggs that they developed from.

Another powerful way to deliver the ecdysteroid receptor gene or anti-sense RNA to the insect *Lucilia cuprina* is by germline transformation. The absence of tools (e.g. transposons) in insects other than *Drosophila* has been a serious obstacle. However, in a recent breakthrough, a new transposon element for making transgenic Drosophila and Medflies has been found (Loukeris et. al., 1995, Science, 270:2002-2005 incorporated herein by reference).

Using this transposon element, germline transformation may be effected by transforming the DNA encoding the ecdysteroid receptor or its antisense DNA according to techniques known in the art. See for example Loukeris et al. referenced above and Oakesshott J., Molecular Approaches to Fundamental and Applied Entomology, Chapter 12: Prospects and Possibilities for Gene Transfer Techniques in Insects, 451-488. In the case of the ecdysteroid receptor DNA, over expression of the ecdysteroid receptor in transgenic flies should prematurely turn on different developmental stages, the intereference with such developmental stages leading to the death of the flies. In the case of the antisense DNA being transformed this should result in low levels of ecdysteroid expression in the transgenic flies preventing the flies advancing to the next developmental stage, also leading to the death of the flies.

This transposon-mediated germline transformation will not only facilitate studies on modulation of blowfly physiology and development by the ecdysteroid receptor, but also facilitate studies and developments, on genetic sexing, sterile-insect techniques and sterilization for sterile male release programs; conferring insecticide resistance to beneficial insects; and increasing insect susceptibility to control agents already in use.

For example, sterile-insect techniques currently employed are often of limited use because of the existence of large quantities of sterile females mating with sterile males and essentially cancelling out the effectiveness of sterile males mating with wild type females. An alternate strategy to reduce the number of viable females would be desirable. One such strategy is to use a female-specific promoter to express an *L. Cuprina* DNA binding domain - estrogen receptor ligand binding domain hybrid protein in females only. In the presence of estrogen, the hybrid protein should activate the *L.Cuprina* receptor and other ecdysone responsive genes. If the estrogen is then added to the fly nutrient medium

during early larval growth, the premature induction of the moulting process should be both female specific and lethal.

Non-limiting examples illustrating the invention will now be provided.

It will be appreciated that the above description is provided by way of example only and variations in both the materials and techniques used which are known to those persons skilled in the art are contemplated.

PROTOCOL

The general strategy of cloning the ecdysteroid receptor was based on reverse transcription (RT) and nested polymerase chain reactions (PCR) and rapid amplification of cDNA ends (RACE) reactions. Figure 1 shows a schematic diagram of the amplification strategy. The first 557 bp fragment covering part of DNA and hormone binding domains of *Lucilia cuprina* ecdysteroid receptor (LcEcR) was amplified by RT-PCR. Degenerate primers 503, 302 and 304 were derived from the regions of DNA and hormone binding domains which are highly conserved among *Drosophila melanogaster* ecdysteroid receptor (DmEcR, Koelle et al., 1991), *Aedes aegypti* ecdysteroid receptor (AaEcR, Cho et al., 1995) and *Chironomus tentans* ecdysteroid receptor (CtEcR, Imhof et al., 1993) sequences. The second amplification of the 1.8 kb fragment spanning the whole hormone binding domain and poly A-tail was accomplished by 3'RACE reactions. The forward sense primers 511 and 512 were derived from the 557 bp sequence information of LcEcR. The putative full length open reading frame of LcEcR was successfully amplified by RT-PCR. The degenerate forward sense primer "ORF1 " was based on DmEcR and CtEcR sequence homology at the very beginning of their open reading frame. The specific reverse antisense primer "3end" was derived from the 1. 8 kilobases (kb) sequence information of LcEcR.

EXAMPLE 1

Preparation of RNA from *Lucilia cuprina* at the late third instar larva

Insects, *Lucilia cuprina* and *Drosophila melanogaster* were reared by standard methods in the Wallaceville Animal Research Centre (WARC) insectary. Total RNA was isolated using TRIzol LS reagent (GIBCO-BRL) with improvement on the original Chomczynski and Sacchi's method (1987).

Four late third instar larvae (0.2 g, 200 µl) of *L. cuprina* or 40 of late third instar larvae of *D. melanogaster* were washed in diethylpyrocarbonate treated H_2O (DEPC-H_2O) to remove sand or food debris etc. Then DEPC-H_2O was added up to the volume of 0.75 mls. Then 2.25 mls of TRIzol LS reagent were added into a 15 ml polypropylene tube and the mixture was homogenised with polytran (Cat. No. IKA 0594000) in an Ultra-turrax T25 homogeniser at 2/3 of maximum speed. The subsequent procedures of phase separation, RNA precipitation, RNA washing and redissolving RNA were followed as in Chomczynski and Sacchi's method.

EXAMPLE 2

RT-PCR amplification of a 557 bp fragment covering part of the DNA binding domain and hormone binding domain

Reverse transcription (RT) and nested polymerase chain reaction (PCR) was employed to amplify a cDNA with 557 bp in size. Degenerate primers 503, 302 and 304 were designed based on DmEcR and AaEcR and CtEcR sequences as shown in Figure 2 and Table 2 below.

Table 2: Primers used to amplify LcEcR

Degenerate primers	(sense) Protein sequence	5' 3' Oligonucleotide sequence
503 (forward)	AVYCCKFG	GCXGTXTAYTGYAARTTYGG
302 (reverse)	DNVEYAL	ARXGCRTAYTCXACRTTRTC
304 (reverse)	QEDQITL	ARXGTDATYTGRTCYTCYTG
ORF1 (forward)	NotIMKRRW	GCGGCCGCATGAARMGXMGXTGG
Specific primers		
511 (forward		CATGCGGCCGGAATGTGTGGTGCCC
512 (forward)		ACAGACCAGTGTGTGTGCAAC
Oligo-dT anchor		GACCACGCGTATCGATGTCGAC$(T)_{16}$V
PCR anchor		GACCACGCGTATCGATGTCGAC
3 end (reverse)		TGTTATTGTTAACATGC

The forward sense primer 503 was based on the region of the DNA-binding domain which is conserved among members of thyroid hormone/retinoic acid receptor family. The reverse antisense primers 302 and 304 were designed based on the hormone-binding domain sequences which share 70-80% homology among the same domains of DmEcR, AaEcR and CtEcR.

cDNA was obtained through reverse transcription with 5 µg of total RNA prepared from whole bodies of blowfly *L. cuprina* at the late 3rd instar larvae stage. Five µg of total RNA with 2 pmols of 302 reverse primer in DEPC-H_2O were added up to 11.2 µl. This mixture was heat-denatured at 100 C for 4 minutes and then quickly chilled on ice and spun down briefly. Then the rest of the following reagents were added to the above RNA and primer mixture to make up the total volume of 19 µl : 4 µl of 5 x first strand buffer,

2 µl of 0.1 M DTT, 1 µl of 10 mM dNTP mix and 0.8 µl (32 units) Rnase-inhibitor (Boehringer Mannheim, Cat. No. 799 017). The contents of the tube were mixed gently and incubated at 42°C for 2 minutes. 1 µl (200 units) of Superscript II reverse transcriptase (GIBCO-BRL, Cat. No. 18064-014) was added and mixed by pipetting gently and then incubated at 42°C for 50 min. The reaction was heat-inactivated at 70°C for 15 min. The cDNA product was used for PCR amplification directly or stored at -20°C if necessary.

Polymerase chain reactions were performed with the Expand High Fidelity PCR system (Boeringer Mannheim, Cat. No. 173261) in a volume of 100 µl using a three separate master mixes protocol. The preparation of three separate master mixes circumvented the need of the hot start. It addition, it avoided the possibility of the enzyme mix interacting with the primer mix and the template mix, since dNTPs could lead to a partial degradation of primer and template through the 3'-5' exonuclease activity of Pwo. Furthermore, the template mix which was added at the last step could avoid potential contamination. The detailed protocol is as follows: In the primer mix, 2 µl of 10 mM dNTPs (final concentration: 200 µM) were mixed with 3 µl of 10 µM forward and reverse primers (final concentration: 300 nM) and sterile redistilled H2O up to 48 µl. Meanwhile, in the enzyme mix, 10 µl of 10 x Expand HF buffer containing 20 mM Tris-HC1, pH 7.5, 100 mM KC1, 1 mM dithiothreitol (DTT), 0.1 mM EDTA, 0.5 % Tween 20 (v/v), 0.5% Nonidet P40 (v/v) and 50% glycerol (v/v) were mixed with 16 µl of 25 mM $MgCl_2$ (final concentration: 4 mM) and 0.75 µls (2.6 units) of Expand High fidelity enzymes and sterile redistilled H_2O was added up to a total volume of 50 µl. Finally, 2 µl of cDNA product (the template mix) were added to 48 µl of the primer mix and 50 µl of the enzyme mix in a 0.2 ml thin-walled tubes. Thermal conditions in a Perkin-Elmer 480 thermal cycler were as follows: one cycle at 94°C for 2 min; 35 cycles at 94°C for 15 s, 55°C for 30 s and 72°C for 1 min. Then 1/10th of the PCR product was electrophoresed in a 1% agarose gel in 50 mM Trisacetate-1 mM EDTA (pH 8.0). DNA bands were visualised by staining with ethidium bromide. Lambda phage DNA digested with HindIII was used for size measurement and quantitation.

The result for this 557 bp fragment amplification is shown in Figure 3. *Drosophila* RNA was used as the positive control. The nested PCR reactions were performed with forward primer 503 and reverse primer 302 as a set for the first run PCR followed by forward primer 503 and reverse primer 304 as a set for the second run PCR. This 557 bp DNA band was then isolated from the low melting agarose gel and in gel ligation was performed with pGEM-T vector (Promega, Cat. No. A3600). The white recombinant pLCm DNA was purified and sent to the Centre for Gene Research, University of Otago for automatic

sequencing. The DNA sequence analysis and its deduced amino acid sequence of this 557 bp fragment was analysed by using the GCG (Genetic Computer Group, University of Wisconsin) database and software. Figure 4 shows the detailed DNA sequence and deduced amino acid sequence.

EXAMPLE 3

3'RACE amplification of the 1.8 kb fragment covering the whole hormone binding domain and poly A tail

The successful amplification of the 557 bp fragment covering part of the DNA and hormone binding domains provided us with tools for subsequent 3'RACE amplification. The forward primers 511 and 512 were derived from this 557 bp sequence information while reverse primers were designed taken advantages of the natural poly (A)-tail of mRNAs. 3' RACE kit was purchased from Boeringer Mannheim (Cat. No. 1734 792). The first strand cDNA synthesis was initiated at the poly (A)-tail of mRNA using the oligo dT-anchor primer. After converting mRNA into cDNA, the nested PCR was performed by 511 and PCR anchor primer as a set for the first run followed by 512 and PCR anchor primer as a set for the second run.

The first strand cDNA synthesis was initiated by oligo dT anchor primer (Table 2) which effectively selected for poly (A)-tailed mRNA. In a microfuge tube, 3.34 µg of *L. cuprina* total RNA with DEPC-H_2O were added up to a total volume of 11.2 µl. It was then heated at 70°C for 10 min and quickly cooled on ice. Then the rest of the reagents were added into the tube: 0.8 µls of RNase inhibitor (40 units /µl), 4 µls of 5 x cDNA synthesis buffer, 2 µls of 10 mM dNTP, 1 µl of 37.5 µM Oligo dT anchor and 1 µl of AMV reverse transcriptase (20 units/ µ1). The mixture were incubated at 55°C for 60 min and then incubated at 65°C for an additional 10 min to inactivate the reverse transcriptase activity. The resulting cDNA product was ready to be used for PCR reaction. PCR reactions were set up in a volume of 50 µl using the 3 master mixes protocol as above, The final primer concentration and dNTP concentrations were 250 nM and 100 µM respectively. The optimisation of $MgCl_2$ concentration was conducted with the series of $MgCl_2$ at 1.0 mM 1.5 mM, 2.75 mM, 4.0 mM and 5.5 mM. It was found that the final concentration of $MgCl_2$ at 1.5 mM resulted in the most optimal outcome. The PCR thermal conditions were as follows: one cycle at 94°C for 2 min; 10 cycles at 94°C for 15 s, 65°C for 30 s and 72°C for 2 min; 25 cycles at 94°C for 15 s, 65°C for 30 s and 72°C for 2 min + additional 20 s elongation for each cycle; one cycle 94°C for 15 s, 65°C for 30 s and 72°C for 7 min.

Amplification of the 1.8 kb fragment spanning the whole hormone binding domain and the poly (A) tail by 3' RACE is shown in Figure 5. The nested PCR reactions were performed with forward primer 511 and reverse PCR anchor primer as a set for the first PCR run followed by forward primer 512 and PCR anchor primer as a set for the second PCR run. This 1.8 kb DNA fragment was then isolated from a 0.8 % agarose gel and subsequently purified with a Prep-A-Gene DNA purification Matrix (Bio-Rad, Cat. No. 732-6011). The ligation between this purified 1.8 kb fragment with pMOSBlue T-vector (Amersham Life Science, Cat. No. RPN 1719) was conducted according to the instructions provided with the pMOSBlue T-vector kit. The recombinant plasmid was designated as pLC3 and the plasmid DNA was purified and sequenced. Figure 4 shows the detailed DNA sequence and its deduced amino acid sequence.

EXAMPLE 4

RT-PCR amplification of the full length open reading frame (2.8 kb) of LcEcR

The 1.8 kb 3'RACE amplification enabled us to design the reverse primer 3'end (Table 2) at the very end of the poly (A) tail from LcEcR. Meanwhile, GCG database analysis showed that DmEcR shares a few amino acid homologies at the very beginning of the open reading frame with AaEcR, but not with CtEcR. If the beginning of the open reading frame of LcEcR shares homology with DmEcR and AaEcR, then the putative full length open reading frame of LcEcR can theoretically be amplified. Thus, the degenerate forward primer ORF1 (Table 2) was designed based on his homology between DmEcR and AaEcR. RT-PCR was used to amplify the putative full length open reading frame of LcEcR. The first stand cDNA synthesis was initiated at the poly (A)-tail of mRNA using the oligo dT-anchor primer. After converting mRNA into cDNA, the nested PCR was performed by ORF1 primer and PCR anchor primer as a set for the first run followed by ORF1 and 3' end primer as a set for the second run.

The first strand cDNA synthesis followed the exact conditions as for the 1. 8 kb LcEcR cDNA synthesis above. PCR reactions were also set up in a volume of 50 µl using the 3 master mixes protocol as well. The final primer concentration and dNTP concentration were 250 nM and 100 µM respectively. The optimisation of MgCl2 concentration was conducted with the series of $MgCl_2$ at 1.0 mM 1.5 mM, 2.75 mM 4.0 mM and 5.5 mM. It was found that a concentration of $MgCl_2$ at 2.75 mM resulted in the most optimal outcome. The PCR thermal conditions were as follows: one cycle at 94°C for 2 min; 10 cycles at 94°C for 15 s, 60°C for 30 s and 72°C for 2 min; 25 cycles at 94°C for 15 s, 60°C for 30 s and 72°C for 2 min + additional 20 s elongation for each cycle; one cycle

94°C for 15 s, 60°C for 30 s and 72°C for 7 min.

The amplification result for the putative full length open reading frame (2.8 kb) is shown in Figure 6. This 2.8 kb DNA fragment was isolated from the 0.8 % agarose gel and subsequently purified by Prep-A-Gene DNA purification Matrix. The ligation between this purified 2.8 kb fragment and pMOSBlue T-vector were conducted according to the manufacturers instructions. The recombinant plasmid was designated as pEcRO and the plasmid DNA was purified and sequenced. Figure 4 shows the detailed DNA sequence and its deduced amino acid sequence.

EXAMPLE 5

Production of Recombinant Baculovirus TnNPV-EcR

The baculovirus gene expression vector system is ideal for the expression of LcEcR as it assures appropriate folding and post-translational modifications of LcEcR. The baculoviral vector system, *Trichoplusia ni* nuclear polyhedrosis virus, TnNPV, developed in Dr. Yi Pang's lab in State Key lab for Biocontrol in Zhongshan University in China (Wang et al., 1991b) has three unique advantages: 1) The recombinant virus (TnVPV-EcR, as shown in Figure 7 is designed to express both the viral polyhedrin-encoding gene and a heterologous gene such as LcEcR. These recombinants form polyhedral occlusion bodies which serve as visible markers of recombination. The recombinants can also be used to facilitate oral infection of insect larvae for mass-scale protein production. 2) The dual promoter, Psyn and Pxiv, which drives LcEcR gene expression, is a much stronger promoter compared with Psyn, Pxiv or Ppolh alone. 3) The β-galactosidase gene expressed by the parenteral polyhedral inclusion body minus vector, EnNPV-SVI-G as shown in Figure 7, can be replaced by the polyhedrin-encoding gene in the recombinant virus. Thus, the recombinant virus TnNPV-EcR shows white phenotype when stained with X-gal. This blue-white color selection can serve as a useful marker for detecting recombinant viruses.

There are four steps involved in generating recombinant baculovirus TnNPV-EcR: 1) Construction of a transfer plasmid containing the LcEcR gene flanking with polyhedrin homologous sequences. 2) Co-transfection of the recombinant transfer plasmid with the parental baculovirus TnNPV-SVI-G. Thus, the LcEcR gene can be transferred into the viral target site by homologous recombination. 3) Selection and plaque-purification of the recombinant virus. 4) Verification of the recombinant virus which directs infected cells to express the LcEcR protein.

The baculovirus transfer plasmid pSXIVVI$^+$X3/2 was chosen as the expression vector for the 2.8 kb LcEcR cDNA subcloning since pSXIVVI$^+$X3/2 contains an efficient translational initiation signal (ATG) and it simplifies the LcEcR cDNA fusion to N-terminal codons in the correct reading frame (Wang et al., 1991a). The resulting plasmid pSXIVVI$^+$X3/2-EcR was cotransfected into Sf-9 cells with the parental polyhedral inclusion body minus vector, TnNPV-SVI$^-$G, which expresses the β-galactosidase gene (Summers and Smith, 1989). After 3-5 runs of plaque purification, three TnNPV-LcEcR-occ+ clones were obtained with the LcEcR gene under the dual control of synthetic and XIV promoters. These three TnNPV-EcR clones all formed polyhedral inclusion bodies in Sf-9 and Tn-5B1 cells and showed white phenotype when stained with X-gal.

To verify that the recombinant baculovirus TnNPV-EcR expressed the LcEcR, a western blot analysis was employed. When the LcEcR cDNA was incorporated into pSXIVVI$^+$X3/2 transfer plasmid, an 11 amino acid peptide (GlnProGluLeuAlaProGluAspProGluAsp) derived from Herpes Simplex virus glycoprotein D was joined in frame to the N-terminal of LcEcR cDNA. Thus, if the mouse monoclonal antibody against HSV-gD epitope (Novogen, Cat.# 69171-1) detected this HSV-gD epitope by western blot, it would indicate LcEcR cDNA was expressed in the right reading frame in the context of the baculovirus vector.

To demonstrate this, after 72 hours post infection, Tn-5B1 cells infected with wild-type TnNPV and TnNPV-EcR were lysed, processed and electrophoresed on a 7.5% SDS-PAGE gel. The gel was then transferred onto a nitrocellulose blotting membrane. The first antibody (anti HSV-gD with 1:5000 dilution) was used and then the second antibody (anti-mouse IgG) was applied to detect HSV-gD epitope. The results are shown in Figure 8 in which the right Lane shows tN-5B1 cells infected with wild-type TnNPV. Left lane shows tn-5B1 cells infected with TnNPV-EcR. More specifically, the left lane shows a polypeptide with a molecular weight in excess of approximately 90 kD as detected by the western blot.

EXAMPLE 6

In vivo functional assay for LcEcR restoring the ecdysone response in an EcR-deficient *Drosophila* cell line

The ecdysteroids bind to an ecdysteroid receptor (EcR) which leads to the formation of a heterodimer with the ultraspiracle protein (USP). This complex activates developmental

genes which contain an ecdysteroid response element (EcRE) (Koolman, 1990, Henrich, 1995). We utilised this feature to design the following trans-activation assay to test whether the LcEcR cDNA we cloned confers ecdysone responsiveness: In an EcR-deficient drosophila Kc line L57-3-11 (derived from *Drosophila melanogaster* (Dm) cell line Kc167, kindly provided by Peter and Lucy Cherbas from Indiana University, USA) (Swevers et al., 1996), its own EcR is deficient in expression by gene-targeting. Thus, the exogenous LcEcR, after transfection into L57-3-11 can form a heterodimer complex with its partner, the endogenous DmUSP. A reporter plasmid containing the β-galactosidase (β-gal) gene controlled by a promoter containing an ecdysteroid response element (EcRE, derived from *Drosophila heat shock protein* 27) can be co-transferred into this line. Therefore, in the presence of 20-hydroxyecdysone, LcEcR/DmUSP forms a specific complex with the EcRE, trans-activiating the β-galactosidase reporter gene expression. β-gal activity can be readily detected with chromogenic or fluorescent reagents.

There are three steps involved in order to set us this *in vivo* function assay: 1)Construction of the recombinant plasmid, pAct/EcR, which carries LcEcR cDNA driven by the *Drosophila* actin 5C promoter. The reporter plasmid pEcRE/Adh/βgal (kindly provided by Dr. Hogness' group) carries β-galactosidase (β-gal) reporter gene driven by Drosophila alcohol dehydrogenase (Adh) promoter containing the EcRE derived from the heat shock protein 27 (*hsp* 27); 2. Cotransfection of the above two plasmids into L57-3-11 cells; 3.Hormonal induction and assay of β-gal in tissue culture cells.

The plasmid pAct/EcR was constructed by inserting a 2.8 kb BamH 1 fragment of LcEcR cDNA from pSXIVVI^{+}X3.2-EcR into the BamH 1 site of pMK26 containing the Drosophila actin 5C promoter (pMK26 was kindly provided by Dr. Hogness' group) (Koelle, et al., 1991). Two plasmids (pAct/EcR and pEcRE/Adh/βgal) were then cotransfected into L-57-3-11 cells by the non-liposomal formulation FuGene 6 transfection reagent (Boehringer-Mannheim, Cat. 1814443) which produces high levels of transfection with minimal cytotoxicity for insect cell lines. L57-3-11 cells were cultured in HyQ CCM3 media (HyClone, Cat. SH30061.03) with 5% bovine fetal serum. Detailed protocol approximating that in the FuGene 6 manual from Boehringer-Mannheim was employed. We found the optimal conditions for transfecting 80% confluency L57-3-11 cells in a 50 ml tissue culture flask to be as follows: 14μg plasmid DNA (11.48 μg of pAct/EcR: 2.1 μg of pMK43.2F) with 12 μl FuGene 6 transfection reagent.

After cotransfection, the ecdysone induction and β-gal activity detection were carried out as follows: 24 hours after transfection, each dish of cells was split in half: one half was treated with 2 x 10^{-6}M of 20-Hydroxyexdysone (Sigma) and another received no hormone

treatment. Cells were then cultured for 24 hours after the addition of the hormone. Tissue culture cells were scraped and spun down at 2000 rpm, for 10 min. Cell pellets were washed once in 1 ml of PBS buffer in an eppendorf tube and then resuspended in 100 µl of freeze/thaw buffer (0.25 M sucrose, 10mM Tris (pH 704), 10mM EDTA). Cells were then repeatedly frozen in liquid nitrogen and thawed in a 37°C water bath for a total of three freeze/thaw cycles. Cell debris was removed by a 10 min centrifugation in a microcentrifuge at 4°C. The concentration of protein in the supernatant (cell extract) was determined by the method of Bradford (1976), with bovine serum albumin as a standard, and typically 1.5-2.5 mg/ml. Extracts were assayed immediately or frozen and assayed up to 2 weeks later with no loss in activity. For 5-10 µl of extract in freeze/thaw buffer containing 0.1 mg/ml bovine serum albumin, 1.5 ml of assay buffer was added (Assay buffer: 0.1 Sodium phosphate (pH 8.0), 5 mM $MgCl_2.6H_2O$, 0.6 mM 4-methylumbelliferyl-β-D-galactoside (dissolve in DMSO first and then add into assay buffer)). After incubation at 37°C for 30 min, the reactions were stopped by adding 1.5 ml of stopping buffer (300 mM Glycine, 15 mM EDTA (pH 11.2)). The fluorescent reaction product was quantified on a Perkin-Elmer Model 2000 Fluorometer, with λex=365 nM and λem=450 nM. β-gal activities were given as fluorescence units per µg of protein assayed.

We tested the ability of LcEcR to mediate hormone-induced transcriptional activation in the EcR-deficient cell line :57-3-11, which contains only on average, 10% of the normal titer of EcR, as measured by iodoponasterone binding. When L57-3-11 cells cotransfected with an LcEcR expression plasmid pAct/EcR and the report plasmid pEcRE/Adh/β-gal, the inducibility of the reporter increased from 7 fold in the absence of LcEcR overexpression to 109 fold in the presence of LcEcR (as shown in Figure 9). This indicated that LcEcR/DmUSP can form a specific complex with the EcRE, *trans*-activating the β-galactosidase reporter gene expression, to restore hormonal inducibility to L57-3-11.

It is to be understood that the scope of the invention is not restricted to the above examples and that numerous variations and modifications may be made to those examples without departing from the scope of the invention as set out in this specification.

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CLAIMS:

1. An isolated *Lucilia cuprina* ecdysteroid receptor (LcEcR) polypeptide which has the amino acid sequence set out in Figure 4, or a variant thereof having substantially equivalent receptor activity thereto.

2. An isolated peptide comprising the amino acid sequence for one or more of receptor domains A/B, C, D, E or F as set out in Figure 4.

3. An isolated peptide consisting of the amino acid sequence for one or more of receptor domains A/B, C, D, E or F as set out in Figure 4.

4. An isolated polypeptide or peptide according to claim 2 or claim 3 wherein the amino acid sequence is the sequence of domain C or E.

5. A nucleic acid molecule encoding an isolated polypeptide according to any one of claims 1 to 4.

6. A nucleic acid molecule according to claim 5 which is selected from the group of RNA, DNA and cDNA molecules.

7. A nucleic acid molecule according to claim 6 which is a DNA molecule.

8. A recombinant expression vector comprising a DNA molecule according to claim 7.

9. A vector according to claim 8 which is a baculovirus vector.

10. A host transformed with a vector according to claim 8 or claim 9.

11. A host according to claim 10 which is an insect cell.

12. An inducible expression system comprising:

(a) a controlling element responsive to a ligand of an ecdysteroid receptor polypeptide or peptide according to any one of claims 1 to 4;

(b) a desired gene for expression, operably linked to the controlling element;

and

(c) an ecdysteroid receptor which can bind to the controlling element.

13. A ligand capable of binding to a polypeptide or peptide according to any one of claims 1 to 4.

14. A ligand according to claim 13 which is an agonist ligand.

15. A ligand according to claim 13 which is an antagonist ligand.

16. A ligand according to claim 13 which is selected from the group consisting of phytoecdysteroids, antibodies, and antibody binding fragments.

17. A method of assaying a sample for the presence of a ligand according to any one of claims 13 to 16, the method comprising:

(a) contacting a sample with a detectably labelled polypeptide or peptide according to any one of claims 1 to 4;

(b) detecting the presence of any bound labelled polypeptides or peptides.

18. A method according to claim 15 wherein the peptide is DNA binding domain C, or hormone binding domain E, as set out in Figure 4.

19. A method for screening for ecdysteroid ligands, the method comprising:

(a) contacting an ecdysteroid responsive element (EcRE), an ecdysteroid receptor (EcR) and a reporter system with a sample;

(b) detecting the presence of any bound, reported ecdysteroid receptor.

20. A method according to claim 19 wherein as a pre-step a cell is transformed with one or more plasmids comprising an ecdysteroid responsive element (EcRE), an ecdysteroid receptor (EcR) and a reporter system. which cell is then contacted with the sample.

21. A method according to claim 20 wherein the cell is an insect cell.

22. A method according to claim 20 wherein the cell is a mammalian cell.

23. A method according to claim 22 wherein the cell does not support ecdysone-responsive transactivation.

24. A method according to any one of claims 19 to 23 wherein the EcRE and EcR are present in different plasmids.

25. A method according to any one of claims 19 to 24 wherein the reporter system comprises a detectable label.

26. A method according to claim 25 wherein the detectable label is a reporter gene.

27. A method according to claim 26 wherein the reporter gene is β-galactosidase.

28. A method according to any one of claims 19 to 27 wherein the EcRE is linked with a promoter.

29. A method according to claim 28 wherein the promoter is an alcohol dehydrogenase promoter.

30. A method according to any one of claims 17 to 29 wherein the sample is a plant extract.

31. A method according to any one of claims 19 to 30 wherein the ligand is an agonist ligand.

32. A method according to any one of claims 19 to 30 wherein the ligand is an agonist ligand.

33. A method according to any one of claims 19 to 32 wherein the method is an *in vivo* method.

34. An insecticidal composition comprising an agriculturally suitable carrier and a vector incorporating a molecule encoding a ecdysteroid receptor (LcEcR) as set forth in Figure 4 or a variant thereof having substantially equivalent activity thereto, or domain C or E thereof.

35. A composition according to claim 34 wherein the vector is a baculovirus.

36. A composition according to claim 35 wherein the baculovirus is a nuclear polyhedrosis virus.

37. A vector comprising a molecule encoding the antisense RNA of a ecdysteroid receptor as set forth in Figure 4, or a variant having substantially equivalent activity thereto, or domain C or E thereof.

38. A vector according to claim 37 which is a baculovirus vector.

39. A vector according to claim 38 which is a nuclear polyhedrosis vector.

40. An insecticidal composition comprising a vector according to any one of claims 37 to 39 or a ligand according to any one of claims 13 to 16 and an agriculturally acceptable carrier.

41. An insect control agent comprising a vector in which a gene encoding an ecdysteroid receptor is inactivated.

42. An agent according to claim 41 in which the vector is a baculovirus vector.

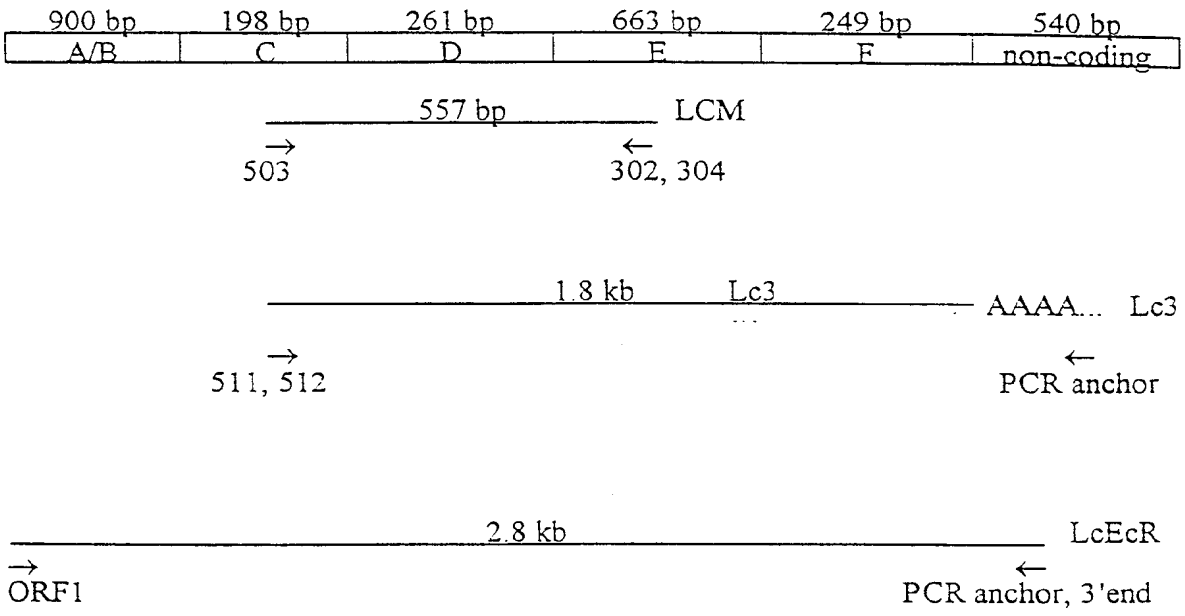
43. A method for controlling insects, the method comprising exposing said insects or larvae thereof to a composition, vector or insecticidal agent as claimed in any one of claims 34 to 42.

44. A method according to claim 43 wherein the insects are flies.

45. A method according to claim 43 or claim 44 wherein the composition, vector or agent is formulated into a bait for administration to adult flies.

46. A method for controlling flies, the method comprising germline transformation of flies using a transposon encoding an ecdysteroid receptor as set forth in Figure 4, or a variant thereof having substantially equivalent activity thereto, or domain C or E thereof.

47. A method according to any one of claims 43 to 46 wherein the flies are *Lucilia cuprina*.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1.8 kb
Lc3
AAAA...
Lc3
511, 512
PCR anchor
2.8 kb
LcEcR
ORF1
PCR anchor, 3'end

FIGURE 1

```
      1                                                            50
DmEcR  .MKRRWSNNG GF..MRLPEE SSSEVTSSSN GLVLPSGVNM SPSSLDSHDY
LcEcR  .MKRRWSNNG GFAALKMLEE SSSEVTSSSN GLVLSSDINM SPSSLDSPVY
AaEcR  MMKRRWSNNG GFTALRMLDD SSSEVTSSSA AL....GMTM SPNSLGSPNY
CtEcR  .......... .......... .......... .......... ..........

       ------>
       primer ORF1

       51                                                          100
DmEcR  CDQDLWLCGN ESGSFGGSNG HGLSQQQQSV ITLAMHGCSS TLPAQTTIIP
LcEcR  GDQEMWLC.N DSASYNNS.. .....HQHSV IT.SLQGCTS SLPAQTTIIP
AaEcR  DELELWSSYE DNA....... ....YNGHSV LSNGNNNLGG CGAANNLLMN
CtEcR  .......... .......... .......... .......... .MKTENLIVT

       101                                                         150
DmEcR  INGNANGNGG STNGQY.... .......... ......VPGA TNLGALA...
LcEcR  LSALPNSNNA SLNNQNQNYQ NGNSMNTNLS VNTNNSVGGG GGGGGVGVPG
AaEcR  GIVGNNNLNG MMNMASQA.. .......... .......... ..........
CtEcR  TVKVEPLNYA SQSFGDNN.. .......... .......... ..........

       151                                                         200
DmEcR  ....NGMLNG GFNGMQQQIQ NGHGLINSTT PSTPTTPLHL QQNLGGAGGG
LcEcR  MTSLNGLGGG GSGSQVNNHN HSHNHLHHNS NSNHSNSHHT NGHMGIGGGG
AaEcR  .......VQA NANSIQHIVG NLINGVNPNQ TLIPPLPSII QNTL......
CtEcR  .......IYG GATKKQRLES D..EWMNHNQ TNMNLESSNM NHNT......

       201                                                         250
DmEcR  G.....IGGM GILHHANGTP NGLIGVVGGG GGVGLGVGGG GVGGLG....
LcEcR  GGLSVNINGP NIVSNAQQLN SLQASQNGQV IHANIGIHSI ISNGLNHHHH
AaEcR  .......... .......... .......... .......... ..........
CtEcR  .......... .......... .......... .......... ..........

       251                                                         300
DmEcR  ........MQ HTPRSDSVNS ISSGRDDLSP SSSLNGYSAN ....ESCDAK
LcEcR  HHMNNSSMMH HTPRSESANS ISSGRDDLSP SSSLNGFSTS ....DASDVK
AaEcR  .........M NTPRSESVNS ISSGREDLSP SSSLNG.... ..YTDGSDAK
CtEcR  .........I SGFSSPDVNY .....EAYSP NSKLDDGNMS VHMGDGLDGK

       301          317                                            350
DmEcR  KS..KKGPAP RVQEELCLVC GDRASGYHYN ALTCEGCKGF FRRSVTKSAV
LcEcR  KI..KKGPAP RLQEELCLVC GDRASGYHYN ALTCEGCKGF FRRSVTKNAV
AaEcR  K..QKKGPTP RQQEELCLVC GDRESGYHYN ALTCEGCKGF FRRSVTKNAV
CtEcR  KSSSKKGPVP RQQEELCLVC GDRASGYHYN ALTCEGCKGF FRRSVTKNAV
                                                        ------>
                                                        primer
```

FIGURE 2/1

```
                                                    382
          351                                        ↓                    400
DmEcR     YCCKFGRACE MDMYMRRKCQ ECRLKKCLAV GMRPECVVPE NQCAMKRREK
LcEcR     YCCKFGHACE MDMYMRRKCQ ECRLEKCLAV GMRPECVVPE NQCAMKRREK
AaEcR     YCCKFGHACE MDMYMRRKCQ ECRLKKCLAV GMRPECVVPE NQCAIKRKEK
CtEcR     YCCKFGHECE MDMYMRRKCQ ECRLKKCLAV GMRPECVVPE NQCAIKRKEK
          ------>
          503

          401                                                        450
DmEcR     KAQKEKDKMT TSPSSQHGGN GSLASGGGQD FV.......K KEILD.LMTC
LcEcR     KAQKEKDKIQ TSVCATE... .......... .I.......K KEILD.LMTC
AaEcR     KAQKEKDKVQ TNATVSTTNS .......... .......TYR SEILPILMKC
CtEcR     KAQKEKDKVP GIVGSNTSSS SLLNQSLNNG SLKNLEISYR EELLEQLMKC

                                                        492
          451                                            ↓          500
DmEcR     EPPQHATIPL LPDEILAKCQ ARNIPSLTYN QLAVIYKLIW YQDGYEQPSE
LcEcR     EPPSHPTCPL LPEDILAKCQ ARNIPPLSYN QLAVIYKLIW YQDGYEQPSE
AaEcR     DPPPHQAIPL LPEKLLQENR LRNIPLLTAN QMAVIYKLIW YQDGYEQPSE
CtEcR     DPPPHPMQQL LPEKLLMENR AKGTPQLTAN QVAVIYKLIW YQDGYEQPSE

        501                                                          550
DmEcR     EDLRRIM.SQ PDENESQTDV SFRHITEITI LTVQLIVEFA KGLPAFTKIP
LcEcR     EDLKRIM.SS PDENESQHDV SFRHITEITI LTVQLIVEFA KGLPAFTKIP
AaEcR     EDLKRIMIGS PNEEEDQHDV HFRHITEITI LTVQLIVEFA KGLPAFTKIP
CtEcR     EDLKRITTEL EEEEDQEHEA NFRYITEVTI LTVQLIVEFA KGLPAFIKIP

          551                                                        600
DmEcR     QEDQITLLKA CSSEVMMLRM ARRYDHSSDS IFFANNRSYT RDSYKMAGMA
LcEcR     QEDQITLLKA CSSEVMMLRM ARRYDHNSDS IFFANNRSYT RDSYKMAGMA
AaEcR     QEDQITLLKA CSSEVMMLRM ARRYDAATDS ILFANNRSYT RDSYRMAGMA
CtEcR     QEDQITLLKA CSSEVMMLRM ARRYDHDSDS ILFANNTAYT KQTYQLAGME
          <------
          primer 304

          601                                                        650
DmEcR     DNIEDLLHFC RQMFSMKVDN VEYALLTAIV IFSDRPGLEK AQLVEAIQSY
LcEcR     DNIEDLLHFC RQMYSMKVDN VEYALLTAIV IFSDRPGLEE AELVEAIQSY
AaEcR     DTIEDLLHFC RQMFSLTVDN VEYALLTAIV IFSDRPGLEQ AELVEHIQSY
CtEcR     ETIDDLLHFC RQMYALSIDN VEYALLTAIV IFSDRPGLEK AEMVDIIQSY
                               <----------
                               primer 302
          651                                                        700
DmEcR     YIDTLRIYIL NRHCGDSMSL VFYAKLLSIL TELRTLGNQN AEMCFSLKLK
LcEcR     YIDTLRIYIL NRHCGDPMSL VFFAKLLSIL TELRTLGNQN AEMCFSLKLK
AaEcR     YIDTLRIYIL NRHAGDPKCS VIFAKLLSIL TELRTLGNQN SEMCFSLKLK
CtEcR     YTETLKVYIV NRHGGESRCS VQFAKLLGIL TELRTMGNKN SEMCFSLKLR

                    713
          701        ↓                                               750
DmEcR     NRKLPKFLEE IWDVHAIPPS VQSHLQITQE ENERLERAER MRASVGGAIT
LcEcR     NRKLPKFLEE IWDVHAIPPS VQSHIQATQA EMA....AQE AQATTSAISA
AaEcR     NRKLPRFLEE IWDVQDIPPS MQAQMHS... .......... .HGTQSSSSS
CtEcR     NRKLPRFLEE VWDVGDVNNQ TTATTNT... .......... .ENIVRERIN
```

FIGURE 2/3

```
        751                                                       800
DmEcR   AGIDCDSAST SAAAAAAQHQ PQPQPQPQPS SLTQNDSQHQ TQPQLQPQLP
LcEcR   AATSSSSINT SMATSSSSSL SPSAASTPNG GAVDYVGTDM SMSLVQSDNA
AaEcR   SSSSSSSSNG SSNGNSSSNS NSSQHGPHPH PHGQQLTPNQ QQHQQQHSQL
CtEcR   RN..... ..... ..... ..... .....

        801                                                       850
DmEcR   PQLQGQLQPQ LQPQLQTQLQ PQIQPQPQLL PVSAPVPASV TAPGSLSAVS
LcEcR   ..... ..... ..... ..... .....
AaEcR   QQVHANGSGS GGGSNNNSSS GGVVPGLGML DQD..... .....
CtEcR   ..... ..... ..... ..... .....
AaEcR   ..... ..... ..... ..... .....
CtEcR   ..... ..... ..... ..... .....

        851                                                       900
DmEcR   TSSEYMGGSA AIGPITPATT SSITAAVTAS STTSAVPMGN GVGVGVGVGG
LcEcR   ..... ..... ..... ..... .....
AaEcR   ..... ..... ..... ..... .....
CtEcR   ..... ..... ..... ..... .....

        901                                          940
DmEcR   NVSMYANAQT AMALMGVALH SHQEQLIGGV AVKSEHSTTA
LcEcR   ..... ..... ..... ..... .....
AaEcR   ..... ..... ..... ..... .....
CtEcR   ..... ..... ..... ..... .....
```

FIGURE 2/3

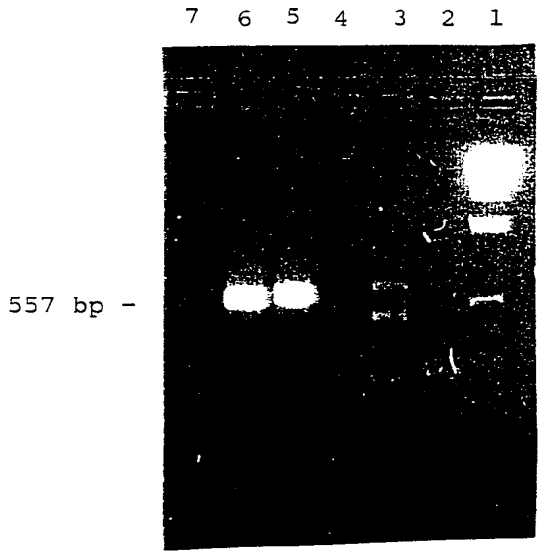
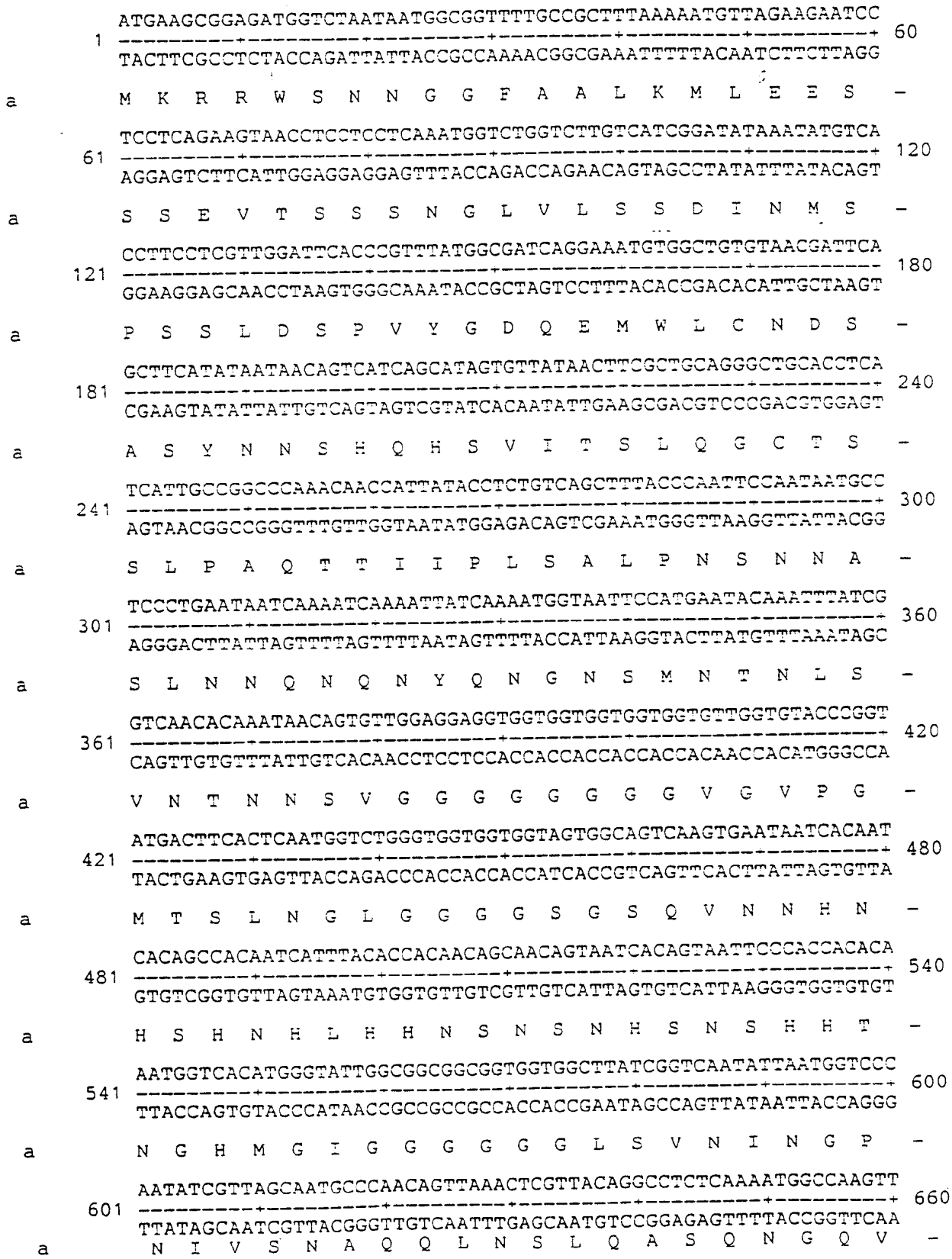


FIGURE 3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61 TCCTCAGAAGTAACCTCCTCCTCAAATGGTCTGGTCTTGTCATCGGATATAAATATGTCA 120
AGGAGTCTTCATTGGAGGAGGAGTTTACCAGACCAGAACAGTAGCCTATATTTATACAGT
a S S E V T S S S N G L V L S S D I N M S -
121 CCTTCCTCGTTGGATTCACCCGTTTATGGCGATCAGGAAATGTGGCTGTGTAACGATTCA 180
GGAAGGAGCAACCTAAGTGGGCAAATACCGCTAGTCCTTTACACCGACACATTGCTAAGT
a P S S L D S P V Y G D Q E M W L C N D S -
181 GCTTCATATAATAACAGTCATCAGCATAGTGTTATAACTTCGCTGCAGGGCTGCACCTCA 240
CGAAGTATATTATTGTCAGTAGTCGTATCACAATATTGAAGCGACGTCCCGACGTGGAGT
a A S Y N N S H Q H S V I T S L Q G C T S -
241 TCATTGCCGGCCCAAACAACCATTATACCTCTGTCAGCTTTACCCAATTCCAATAATGCC 300
AGTAACGGCCGGGTTTGTTGGTAATATGGAGACAGTCGAAATGGGTTAAGGTTATTACGG
a S L P A Q T T I I P L S A L P N S N N A -
301 TCCCTGAATAATCAAAATCAAAATTATCAAAATGGTAATTCCATGAATACAAATTTATCG 360
AGGGACTTATTAGTTTTAGTTTTAATAGTTTTACCATTAAGGTACTTATGTTTAAATAGC
a S L N N Q N Q N Y Q N G N S M N T N L S -
361 GTCAACACAAATAACAGTGTTGGAGGAGGTGGTGGTGGTGGTGTTGGTGTACCCGGT 420
CAGTTGTGTTTATTGTCACAACCTCCTCCACCACCACCACCACAACCACATGGGCCA
a V N T N N S V G G G G G G G V G V P G -
421 ATGACTTCACTCAATGGTCTGGGTGGTGGTGGTAGTGGCAGTCAAGTGAATAATCACAAT 480
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a M T S L N G L G G G G S G S Q V N N H N -
481 CACAGCCACAATCATTTACACCACAACAGCAACAGTAATCACAGTAATTCCCACCACACA 540
GTGTCGGTGTTAGTAAATGTGGTGTTGTCGTTGTCATTAGTGTCATTAAGGGTGGTGTGT
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541 AATGGTCACATGGGTATTGGCGGCGGCGGTGGTGGCTTATCGGTCAATATTAATGGTCCC 600
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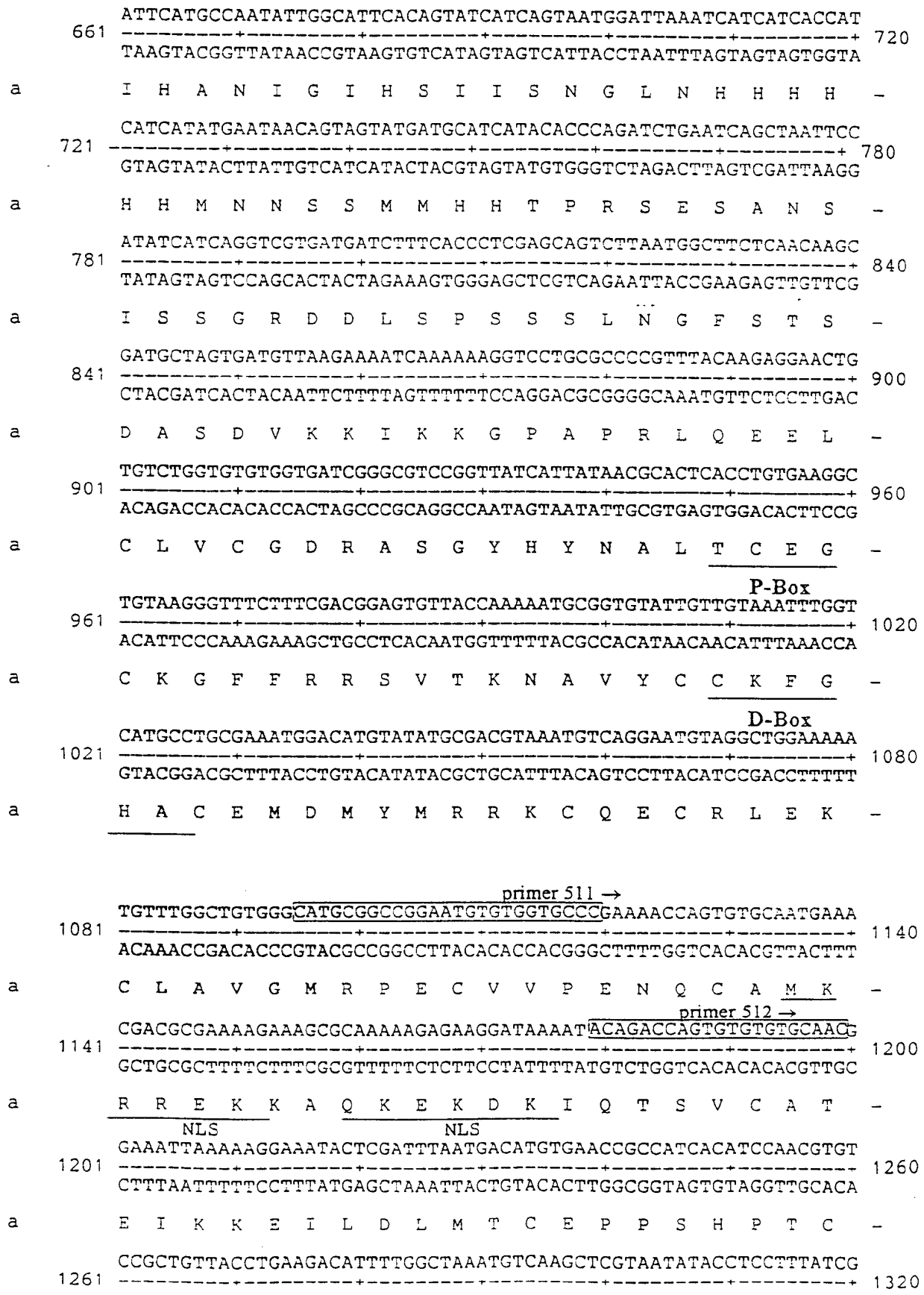
FIGURE 4/1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721
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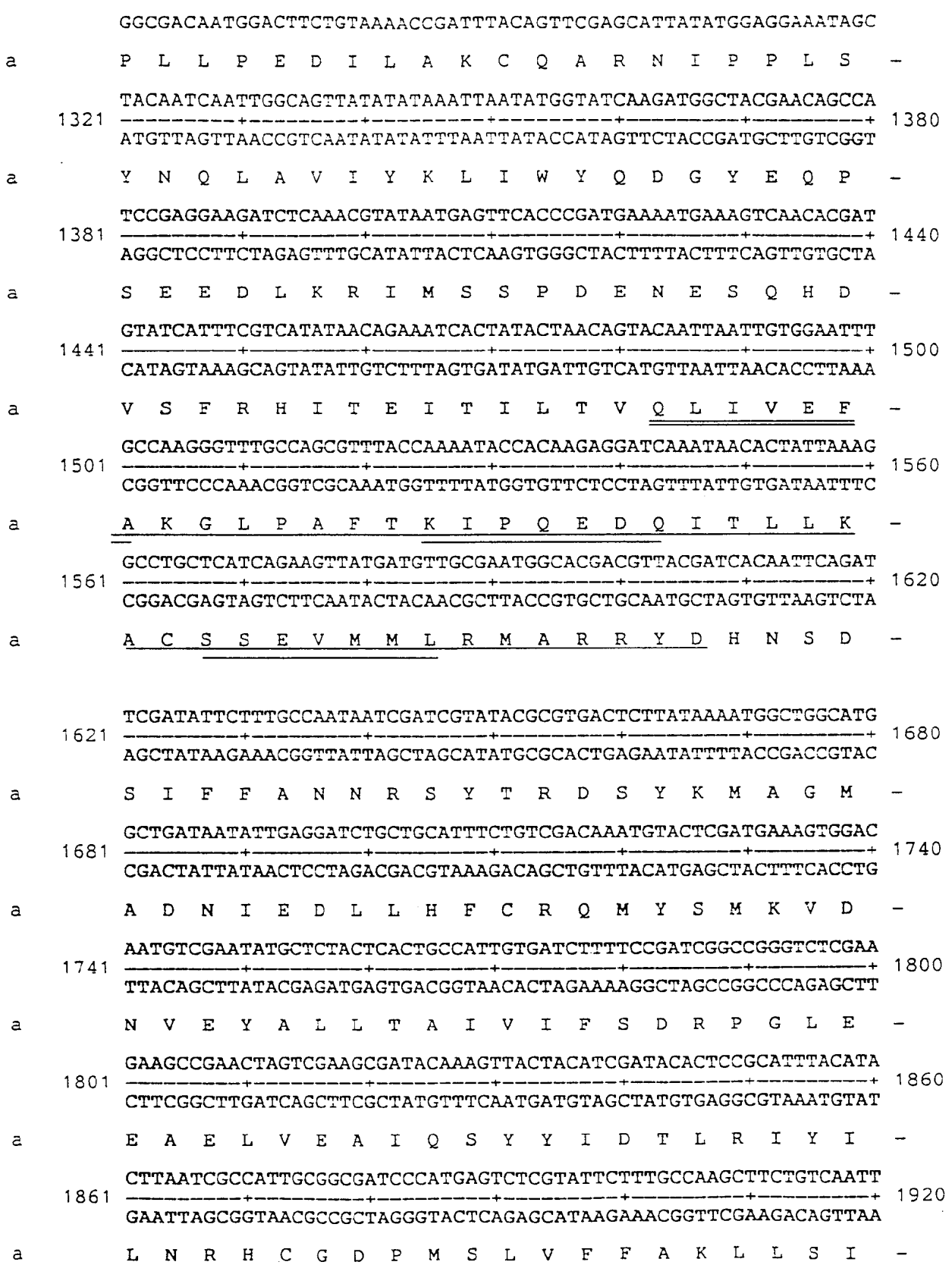
961
1020
ACATTCCCAAAGAAAGCTGCCTCACAATGGTTTTTACGCCACATAACAACATTTAAACCA
a C K G F F R R S V T K N A V Y C C K F G -
D-Box
CATGCCTGCGAAATGGACATGTATATGCGACGTAAATGTCAGGAATGTAGGCTGGAAAAA
1021
1080
GTACGGACGCTTTACCTGTACATATACGCTGCATTTACAGTCCTTACATCCGACCTTTTT
a H A C E M D M Y M R R K C Q E C R L E K -
primer 511 →
TGTTTGGCTGTGGGCATGCGGCCGGAATGTGTGGTGCCCGAAAACCAGTGTGCAATGAAA
1081
1140
ACAAACCGACACCCGTACGCCGGCCTTACACACCACGGGCTTTTGGTCACACGTTACTTT
a C L A V G M R P E C V V P E N Q C A M K -
primer 512 →
CGACGCGAAAAGAAAGCGCAAAAAGAGAAGGATAAAATACAGACCAGTGTGTGTGCAACG
1141
1200
GCTGCGCTTTTCTTTCGCGTTTTTCTCTTCCTATTTTATGTCTGGTCACACACACGTTGC
a R R E K K A Q K E K D K I Q T S V C A T -
NLS
NLS
GAAATTAAAAAGGAAATACTCGATTTAATGACATGTGAACCGCCATCACATCCAACGTGT
1201
1260
CTTTAATTTTTCCTTTATGAGCTAAATTACTGTACACTTGGCGGTAGTGTAGGTTGCACA
a E I K K E I L D L M T C E P P S H P T C -
CCGCTGTTACCTGAAGACATTTTGGCTAAATGTCAAGCTCGTAATATACCTCCTTTATCG
1261
1320

FIGURE 4/2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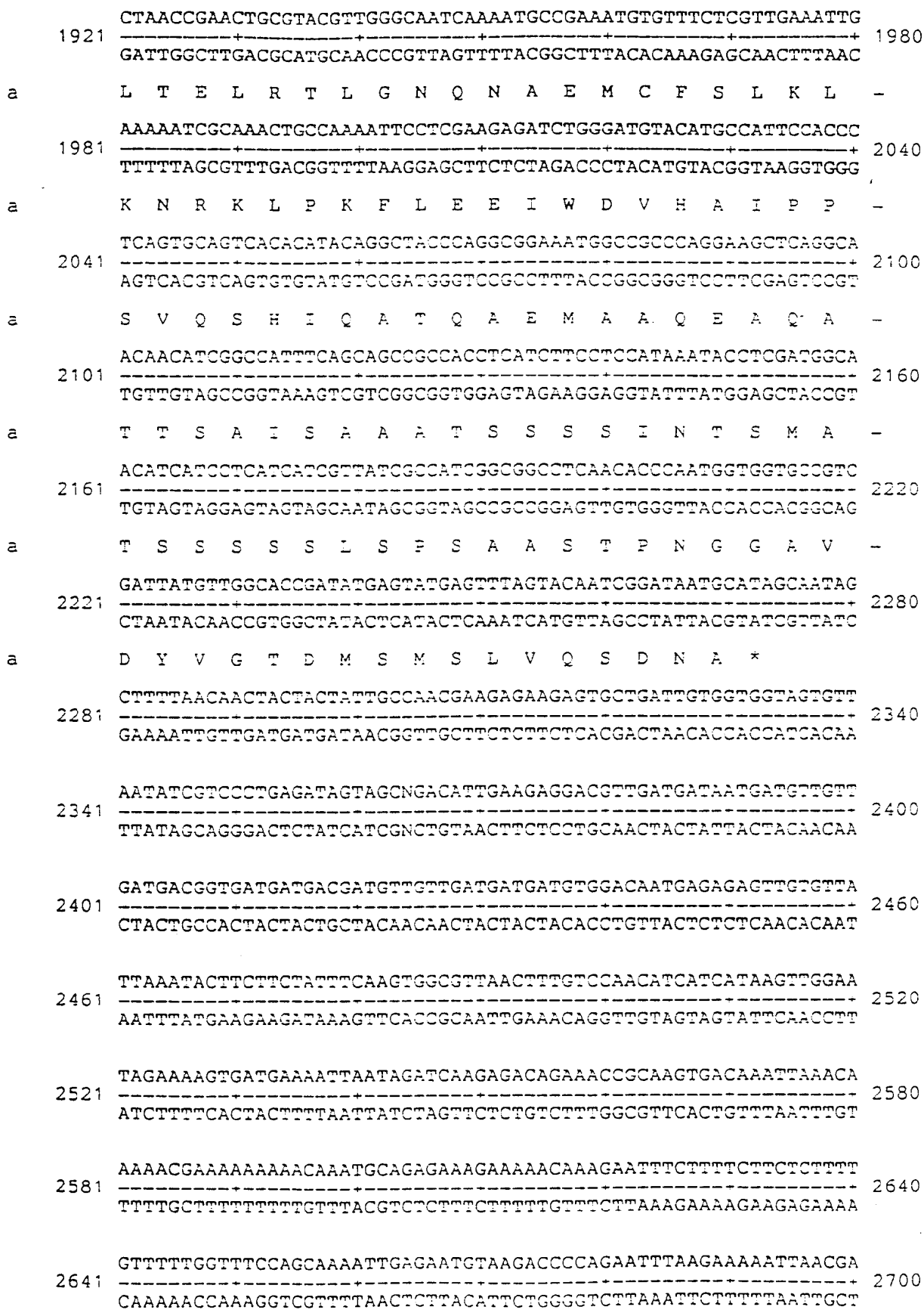
a P L L P E D I L A K C Q A R N I P P L S -
TACAATCAATTGGCAGTTATATATAAATTAATATGGTATCAAGATGGCTACGAACAGCCA
1321 1380
ATGTTAGTTAACCGTCAATATATATTTAATTATACCATAGTTCTACCGATGCTTGTCGGT
a Y N Q L A V I Y K L I W Y Q D G Y E Q P -
TCCGAGGAAGATCTCAAACGTATAATGAGTTCACCCGATGAAAATGAAAGTCAACACGAT
1381 1440
AGGCTCCTTCTAGAGTTTGCATATTACTCAAGTGGGCTACTTTTACTTTCAGTTGTGCTA
a S E E D L K R I M S S P D E N E S Q H D -
GTATCATTTCGTCATATAACAGAAATCACTATACTAACAGTACAATTAATTGTGGAATTT
1441 1500
CATAGTAAAGCAGTATATTGTCTTTAGTGATATGATTGTCATGTTAATTAACACCTTAAA
a V S F R H I T E I T I L T V Q L I V E F -
GCCAAGGGTTTGCCAGCGTTTACCAAAATACCACAAGAGGATCAAATAACACTATTAAAG
1501 1560
CGGTTCCCAAACGGTCGCAAATGGTTTTATGGTGTTCTCCTAGTTTATTGTGATAATTTC
a A K G L P A F T K I P Q E D Q I T L L K -
GCCTGCTCATCAGAAGTTATGATGTTGCGAATGGCACGACGTTACGATCACAATTCAGAT
1561 1620
CGGACGAGTAGTCTTCAATACTACAACGCTTACCGTGCTGCAATGCTAGTGTTAAGTCTA
a A C S S E V M M L R M A R R Y D H N S D -
TCGATATTCTTTGCCAATAATCGATCGTATACGCGTGACTCTTATAAAATGGCTGGCATG
1621 1680
AGCTATAAGAAACGGTTATTAGCTAGCATATGCGCACTGAGAATATTTTACCGACCGTAC
a S I F F A N N R S Y T R D S Y K M A G M -
GCTGATAATATTGAGGATCTGCTGCATTTCTGTCGACAAATGTACTCGATGAAAGTGGAC
1681 1740
CGACTATTATAACTCCTAGACGACGTAAAGACAGCTGTTTACATGAGCTACTTTCACCTG
a A D N I E D L L H F C R Q M Y S M K V D -
AATGTCGAATATGCTCTACTCACTGCCATTGTGATCTTTTCCGATCGGCCGGGTCTCGAA
1741 1800
TTACAGCTTATACGAGATGAGTGACGGTAACACTAGAAAAGGCTAGCCGGCCCAGAGCTT
a N V E Y A L L T A I V I F S D R P G L E -
GAAGCCGAACTAGTCGAAGCGATACAAAGTTACTACATCGATACACTCCGCATTTACATA
1801 1860
CTTCGGCTTGATCAGCTTCGCTATGTTTCAATGATGTAGCTATGTGAGGCGTAAATGTAT
a E A E L V E A I Q S Y Y I D T L R I Y I -
CTTAATCGCCATTGCGGCGATCCCATGAGTCTCGTATTCTTTGCCAAGCTTCTGTCAATT
1861 1920
GAATTAGCGGTAACGCCGCTAGGGTACTCAGAGCATAAGAAACGGTTCGAAGACAGTTAA
a L N R H C G D P M S L V F F A K L L S I -

FIGURE 4/3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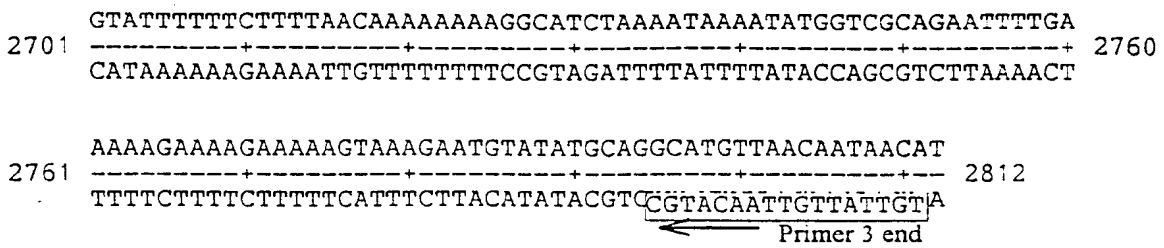
GATTGGCTTGACGCATGCAACCCGTTAGTTTTACGGCTTTACACAAAGAGCAACTTTAAC
a L T E L R T L G N Q N A E M C F S L K L -
1981 AAAAATCGCAAACTGCCAAAATTCCTCGAAGAGATCTGGGATGTACATGCCATTCCACCC 2040
TTTTTAGCGTTTGACGGTTTTAAGGAGCTTCTCTAGACCCTACATGTACGGTAAGGTGGG
a K N R K L P K F L E E I W D V H A I P P -
2041 TCAGTGCAGTCACACATACAGGCTACCCAGGCGGAAATGGCCGCCCAGGAAGCTCAGGCA 2100
AGTCACGTCAGTGTGTATGTCCGATGGGTCCGCCTTTACCGGCGGGTCCTTCGAGTCCGT
a S V Q S H I Q A T Q A E M A A Q E A Q A -
2101 ACAACATCGGCCATTTCAGCAGCCGCCACCTCATCTTCCTCCATAAATACCTCGATGGCA 2160
TGTTGTAGCCGGTAAAGTCGTCGGCGGTGGAGTAGAAGGAGGTATTTATGGAGCTACCGT
a T T S A I S A A A T S S S S I N T S M A -
2161 ACATCATCCTCATCATCGTTATCGCCATCGGCGGCCTCAACACCCAATGGTGGTGCCGTC 2220
TGTAGTAGGAGTAGTAGCAATAGCGGTAGCCGCCGGAGTTGTGGGTTACCACCACGGCAG
a T S S S S S L S P S A A S T P N G G A V -
2221 GATTATGTTGGCACCGATATGAGTATGAGTTTAGTACAATCGGATAATGCATAGCAATAG 2280
CTAATACAACCGTGGCTATACTCATACTCAAATCATGTTAGCCTATTACGTATCGTTATC
a D Y V G T D M S M S L V Q S D N A *
2281 CTTTTAACAACTACTACTATTGCCAACGAAGAGAAGAGTGCTGATTGTGGTGGTAGTGTT 2340
GAAAATTGTTGATGATGATAACGGTTGCTTCTCTTCTCACGACTAACACCACCATCACAA
2341 AATATCGTCCCTGAGATAGTAGCNGACATTGAAGAGGACGTTGATGATAATGATGTTGTT 2400
TTATAGCAGGGACTCTATCATCGNCTGTAACTTCTCCTGCAACTACTATTACTACAACAA
2401 GATGACGGTGATGATGACGATGTTGTTGATGATGATGTGGACAATGAGAGAGTTGTGTTA 2460
CTACTGCCACTACTACTGCTACAACAACTACTACTACACCTGTTACTCTCTCAACACAAT
2461 TTAAATACTTCTTCTATTTCAAGTGGCGTTAACTTTGTCCAACATCATCATAAGTTGGAA 2520
AATTTATGAAGAAGATAAAGTTCACCGCAATTGAAACAGGTTGTAGTAGTATTCAACCTT
2521 TAGAAAAGTGATGAAAATTAATAGATCAAGAGACAGAAACCGCAAGTGACAAATTAAACA 2580
ATCTTTTCACTACTTTTAATTATCTAGTTCTCTGTCTTTGGCGTTCACTGTTTAATTTGT
2581 AAAACGAAAAAAAAACAAATGCAGAGAAAGAAAAACAAAGAATTTCTTTTCTTCTCTTTT 2640
TTTTGCTTTTTTTTTGTTTACGTCTCTTTCTTTTTGTTTCTTAAAGAAAAGAAGAGAAAA
2641 GTTTTTGGTTTCCAGCAAAATTGAGAATGTAAGACCCCAGAATTTAAGAAAAATTAACGA 2700
CAAAAACCAAAGGTCGTTTTAACTCTTACATTCTGGGGTCTTAAATTCTTTTTAATTGCT

FIGURE 4/4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2701
2760
CATAAAAAAAGAAAATTGTTTTTTTTCCGTAGATTTTATTTTATACCAGCGTCTTAAAACT
AAAAGAAAAGAAAAAGTAAAGAATGTATATGCAGGCATGTTAACAATAACAT
2761
2812
TTTTCTTTTCTTTTTCATTTCTTACATATACGTCCGTACAATTGTTATTGTA
Primer 3 end

FIGURE 4/5

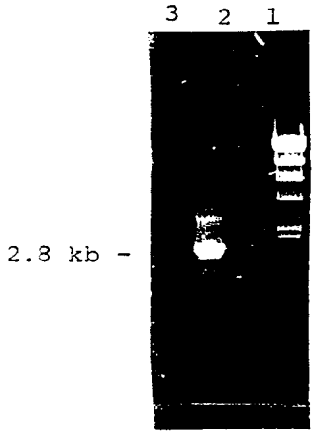


FIGURE 5

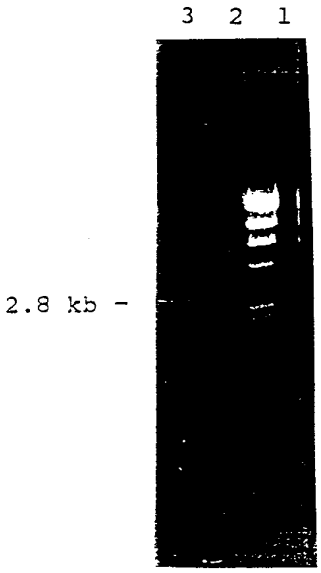


FIGURE 6

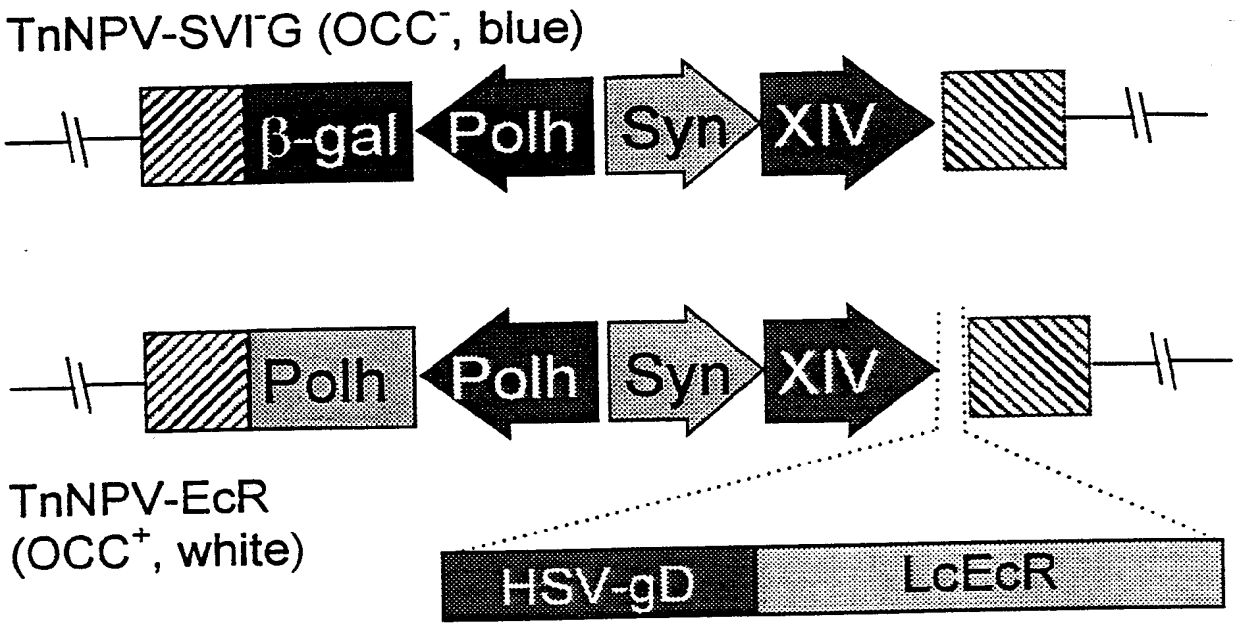


FIGURE 7

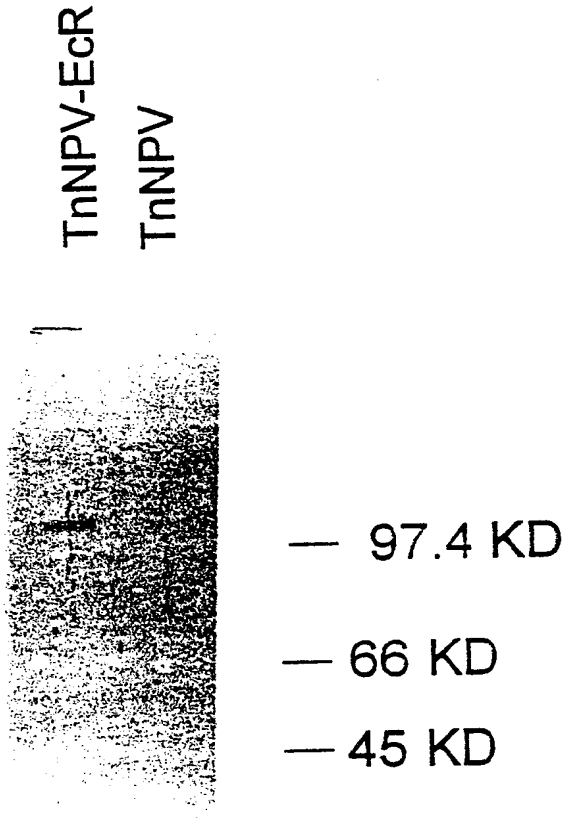
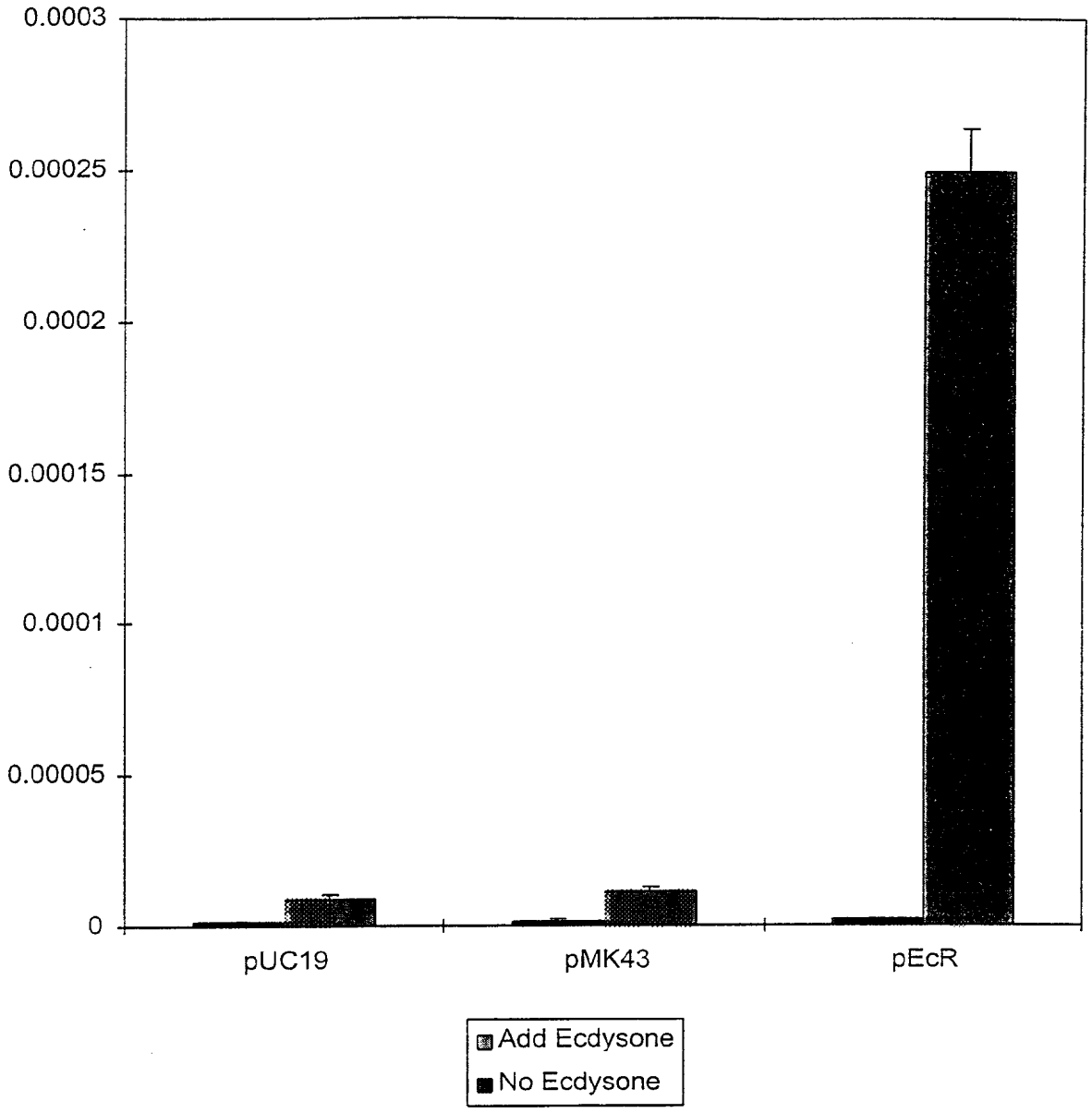


FIGURE 8



Fold induction by ecdysone	9X	7X	109X

Figure 9.